THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicant:	Habib Zaghouani
Appl. No.:	10/681,788
Conf. No.:	6701
Filed:	October 8, 2003
Title:	SUSTAINED TREATMENT OF TYPE 1 DIABETES AFTER EXPRESSION OF PREDISPOSITION MARKERS
Art Unit:	1644
Examiner:	Edwoldt, G.R.
Docket No.:	3718027.00005

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPELLANTS' APPEAL BRIEF

Sir:

Appellants submit this Appeal Brief in support of the Notice of Appeal filed on October 31, 2008. This Appeal is taken from the Final Rejection in the Office Action dated October 8, 2008 and the Notice of Non-Compliant Appeal Brief dated December 31, 2009.

I. REAL PARTIES IN INTEREST

The real parties in interest for the above-identified patent application on Appeal is The Curators of The University of Missouri by virtue of Assignments recorded on November 21. 2006 at reel/frame 018542/0168 in the United States Patent and Trademark Office.

II. RELATED APPEALS AND INTERFERENCES

Appellant's legal representative and the Assignees of this patent application do not know of any prior or pending appeals, interferences or judicial proceedings that may be related to, directly affect or be directly affected by or have a bearing on the Board's decision with respect to the above-identified Appeal.

III. STATUS OF CLAIMS

Claims 1 – 5, 7, 13, 15-19, and 22-30 are pending in this application. Claim 6 was previously cancelled and claims 8 – 12, 20 and 21 are withdrawn. Claims 1 – 5, 7, 13, 15-19, and 22-30 stand rejected. Therefore, 1 – 5, 7, 13, 15-19, and 22-30 (with claim 1 in independent form) are being appealed in this Brief. The appealed claims are reproduced in the Claims Appendix.

IV. STATUS OF AMENDMENTS

A final Office Action was mailed on October 8, 2008. Applicants filed a Notice of Appeal and Pre-Appeal Brief Request for Review on October 31, 2008. A Notice of Panel Decision from Pre-Appeal Brief Review was mailed on June 17, 2009. The Panel Decision sent this matter directly to the Board of Patent Appeals and Interferences. A copy of the final Office Action is attached as Exhibit A, a copy of the Pre-Appeal Brief Request for Review is attached as Exhibit B, and a copy of the Notice of Panel Decision is attached as Exhibit C.

V. SUMMARY OF CLAIMED SUBJECT MATTER

A summary of the claimed subject matter by way of reference to the specification and/or figures for sole independent claim 1 is provided as follows:

A method of preventing or delaying onset of Type 1 diabetes in a subject in need thereof (Page 4, lines 11-14), the method comprising administering to the subject a pharmaceutically acceptable composition comprising a soluble fusion protein (page 25, line 9), wherein the fusion protein comprises at least one immunoglobulin having a variable region comprising a CDR1, a CDR2, or a CDR3 region (page 22, lines 23-24), the at least one immunoglobulin having at least one protein fragment or peptide inserted within the variable region (page 22, lines 19 – 20); wherein (a) the protein fragment or peptide is GAD2 represented by SEQ. ID NO 4 (page 23, line 1), (b) the subject has undergone insulin autoantibody seroconversion prior to said administering step (page 21, line 10 and page 27 line 21- page 28, line 2) and (c) the composition is administered to the subject in one or more dosage administrations (page 37, lines 21-22 and original claim 1).

Although citations are given in accordance with 37 C.F.R. § 41.37(v), these reference numerals and citations are merely examples of support in the specification for the terms used in this section of the Brief. There is no intention to suggest in any way that the terms of the claims are limited to the examples in the specification. As demonstrated by the references numerals and citations, the claims are fully supported by the specification as required by law. However, it is improper under the law to read limitations from the specification into the claims. Pointing out specification support for the claim terminology in accordance with Rule 41.37(v) does not in any way limit the scope of the claims to those examples from which they find support. Nor does this exercise provide a mechanism for circumventing the law precluding reading limitations into the claims from the specification. In short, the references numerals and specification citations are not to be construed as claim limitations or in any way used to limit the scope of the claims.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Whether claims 1-5, 7, 13, 15-19, 22-26 and 27-30 are unpatentable under 35 U.S.C. §112, first paragraph as containing subject matter which was not described in the specification in such way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

2. Whether claims 1, 2, 4, 5, 7, 13, 15-19, 22-24 and 28-30 are unpatentable under 35 U.S.C. § 103(a) as being obvious over WO 98/30706 in view of Kaufman et al., J. Clin. Invest. Vol 89, (1992) 283-292 ("*Kaufman*"). Copies of WO 98/30706 and *Kaufman* are attached hereto as Exhibits D and E, respectively, in the Evidence Appendix.

3. Whether claims 1- 5, 7, 13, 15-19 and 22-30 are unpatentable under 25 U.S.C. § 112, first paragraph as failing to comply with the written description requirement.

4. Whether claims 1- 5, 7, 13, 15-19, 22-25 and 27-30 are unpatentable under the judicially created doctrine of obviousness type double patenting over claims 1 – 7 and 13 – 16 of U.S. 11/290,070.

5. Whether claims 1- 5, 7, 13, 15-19, 22-25 and 27-30 are unpatentable under the judicially created doctrine of obviousness type double patenting over claims 1 – 7 and 13 – 16 of U.S. 11/425,084.

VII. ARGUMENT

1. REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH SHOULD BE REVERSED BECAUSE THE EXAMINER HAS FAILED TO ESTABLISH A PRIMA FACIE CASE OF LACK OF ENABLEMENT OR APPLICANT HAS REBUTTED ANY SUCH PRIMA FACIE CASE.

Claims 1-5, 7, 13, 15-19, 22-26 and 27-30 stand rejected under 35 U.S.C. §112, first paragraph as allegedly containing subject matter which was not described in the specification in such way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. As described in detail below, no *prima facie* case of lack of enablement has been established. Furthermore, even if a *prima facie* case of lack of enablement has been established, which is denied, Applicants previously rebutted it.

A. No *prima facie* case.

It is settled law that a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enablement requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein. MPEP 2164.04 citing *In re Marzocchi*, 58 C.C.P.A. 1069, 439 F.2d 220, 223, 169 U.S.P.Q. (BNA) 367, 369 (CCPA 1971). Thus, the PTO has the initial burden of challenging a presumptively correct assertion of enablement in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. *See In re Bundy*, 642 F.2d 430, 433, 209 U.S.P.Q. (BNA) 48, 51 (CCPA 1981).

The PTO has not provided any credible evidence showing that one of ordinary skill in the art would ***reasonably doubt*** the asserted utility of the claimed invention and has therefore not met its initial burden. The final Office Action dated October 8, 2008 (Exhibit A) at page 3 states that:

> [w]hile the mechanism of action for the method of the instant claims is not disclosed, it appears to require inducing tolerance to GAD and altered GAD 'derived' peptides. Tolerance-inducing peptide immunotherapy is well known in the immunological arts. In some cases significant results have been demonstrated in in-bred small animal models. However said results have not been repeated in human trials.

As an initial matter, Applicants point out that tolerance induction as a mechanism of action is not a limitation of the present claims. Furthermore, the final Office Action only points to 3 references to supports its conclusion. As discussed in detail below, the sum total the evidence shows that, using fundamentally different therapeutic agents than presently claimed, tested in diseases other than Type 1 diabetes as presently claimed, some researchers have achieved tolerance results in animal models that have been difficult to reproduce in humans. See Office Action dated 10/08/2008 at page 3. The first reference relied on—Marketletter Pubs (UK) 13 September 1999 ("*Marketletter*" EXHIBIT F)—deals with two unsubstituted peptides, which are much different than the claimed Ig-GAD2 fusion protein, tested in Multiple Sclerosis and Rheumatoid Arthritis, which are different autoimmune disorders having different initiating autoantigens and different etiologies as compared with Type 1 diabetes. The second reference, Anderton, S.M., Immunology. 2001; 104:367-376 ("*Anderton*" EXHIBIT G), is a review article generally discussing administration of native antigen and analogue peptides in a Multiple Sclerosis model which, again, is a much different therapeutic modality than the claimed fusion protein and a different disease state than Type 1 diabetes. Finally, the third reference, Dong, V.M. *et al.*, Ped. Transplan. 1999; 3:181-192 ("*Dong*" EXHIBIT H), is merely a general review of tissue graft transplant tolerance (unrelated to treatment of any autoimmune disorder let alone Type 1 diabetes) and contains nothing to call in to question use of any fusion protein construct for delaying or preventing Type 1 diabetes. At most, these references are only tangentially related to the presently claimed invention and simply do not cast any doubt, let alone any reasonable doubt, on the presently clamed invention which entails an altogether different therapeutic agent and altogether different disease state than those discussed in the references relied upon by the PTO. This is simply not enough to establish a *prima facie* case of lack of enablement.

Based on the logic used in the instant rejection, the use of a novel compound to treat a given cancer would be unpatentable in the absence of human data if prior treatment of an

altogether different cancer with a different compound had shown success in animals but failed to achieve FDA approval. This is neither the law nor sound policy. Applicants respectfully submit that the burden of challenging the presumptively correct assertion of the manner of making and using the invention has not been met.

Furthermore, Applicants point out that the specification provides substantial guidance as to how to practice the claimed invention. For example:

- Guidance as to how to make the claimed constructs is provided at pages 45, line 13 – page 47, line 3;
- General dosing guidance is provided at page 34, line 21- page 37, line 17;
- Guidance on how to determine whether administration of a claimed fusion protein effectively prevented or delayed diabetes in humans or mice is provided at page 42, line 3 – page 42, line 7 and page 45, lines 5 – 11;
- Guidance on how to determine if a subject has undergone insulin autoantibody seroconversion is provided at page 55, line 15 – page 56, line 24; and
- A working example of delay of onset of diabetes using the general approach claimed (with an Ig-INSβ construct) is provided at page 57, line 19 – page 58, line 5.

For at least the foregoing reasons, no *prima facie* case of lack of enablement has been established. Reversal of this rejection is therefore respectfully requested.

B. Even assuming, *arguendo*, that a *prima facie* case exists, Applicants previously rebutted it.

To further demonstrate that Applicants' claimed invention was enabled at the time of filing, Applicants previously submitted a declaration under 37 CFR 1.312 showing that the claimed method effectively prevents and/or delays the onset of Type 1 diabetes in the gold standard NOD mouse model for that disease. ("Zaghouani Declaration" EXHIBIT I).

i. Post-filing date data can be relied on to demonstrate enablement at the time of filing.

The final Office Action states that "the work [summarized in the Declaration] was apparently done after the effective filing date and therefore cannot be relied upon to show enablement at the time of filing as is required" (EXHIBIT A at page 4, last paragraph – page 5, first paragraph; emphasis added). This statement is legally erroneous. Contrary to the

Examiner's position, both the MPEP and Federal Circuit case law are clear that post-filing date declarations can be used to demonstrate that the claimed invention was enabled when filed and must be considered when submitted. MPEP 2164.05 and *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995).

The *Brana* case is particularly on point. In *Brana*, the Federal Circuit stated in the context of a §112, first paragraph enablement rejection that "[e]ven if one skilled in the art would have reasonably questioned the asserted utility, *i.e.*, even if the PTO met its initial burden thereby shifting the burden to the applicants to offer rebuttal evidence, applicants proffered sufficient evidence to convince one of skill in the art of the asserted utility." *Id* at 1567. In *Brana*, the applicants provided a post-filing date declaration showing that compounds within the scope of the claims exhibited significant anti-tumor activity against the L1210 standard tumor model *in vivo*. According to the court, such evidence alone should have been sufficient to satisfy applicants' burden. The court noted that "[t]he Kluge declaration, though dated after applicant's filing date, could be used to substantiate any doubts as to the asserted utility since this pertains to the accuracy of a statement already in the specification. It does not render an insufficient disclosure enabling, but instead goes to prove that the disclosure was in fact enabling when filed (i.e., demonstrated utility)." (internal citation omitted).

Like the declaration submitted in *Brana*, the Zaghouani Declaration submitted in the instant case also pertains to the accuracy of statements already in the specification and evidences that the original disclosure was in fact enabling when filed. Specifically, the Zaghouani Declaration illustrates the accuracy of disclosure in the specification (*e.g.* pages 45-46 and elsewhere) that the claimed soluble Ig-GAD2 construct is useful for preventing and/or delaying onset of Type 1 diabetes.

ii. Treatment of humans is not required.

The Examiner further takes the position that "while the invention might delay onset of diabetes in some experimental mice…it is not enabled for the prevention of disease in any species nor the preventing or delay of disease in humans." (EXHIBIT A at page 4 and 6). The Examiner attempts to support this conclusion by citing to instances in which treatment (albeit again with fundamentally different types of therapeutic agents and in different diseases than Type 1 diabetes as discussed above) succeeded in animals but failed in humans—for example

Marketletter (EXHIBIT F). That position—also rejected by the Federal Circuit in *Brana*—is clear legal error.

In *Brana*, the PTO argued in the context of a 112, first paragraph rejection that *in vivo* test results in animals are not reasonably predictive of the success of the claimed compounds for treating cancer in humans. *Id* at 20. In response, the Federal Circuit stated:

> The Commissioner, as did the Board, confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption... proof of an alleged pharmaceutical property for a compound by statistically significant tests with standard experimental animals is sufficient to establish utility. *In re Krimmel*, 48 C.C.P.A. 1116, 292 F.2d 948, 953...In concluding that similar *in vivo* tests were adequate proof of utility the court in *In re Krimmel* stated: We hold as we do because it is our firm conviction that one who has taught the public that a compound exhibits some desirable pharmaceutical property **in a standard experimental animal has made a significant and useful contribution to the art, even though it may eventually appear that the compound is without value in the treatment of humans**. *Id*. At 22. (emphasis added)

As has been clearly established in the record, the NOD mouse model used in the experiment described in the Zaghouani Declaration is considered the gold standard animal model for Type 1 diabetes (EXHIBIT J). It was clear error to reject the instant claims under 35 U.S.C. § 112, first paragraph on the alleged basis that successful results in the gold standard animal model for Type 1 diabetes do not necessarily translate to humans or other species. Applicants have taught the public that the claimed soluble IgGAD2 construct can prevent or delay onset of Type 1 diabetes in a standard experimental animal and have thus made a significant and useful contribution to the art, even though it could eventually be determined that the compound is without value in the treatment of humans.

For at least these reasons, no *prima facie* case of lack of written description has been established. Even if a *prima facie* case of lack of written description is deemed to have been established, which is not admitted, Applicants have rebutted it. Reversal of this rejection is therefore respectfully requested.

2. REJECTION UNDER 35 U.S.C. §103(A) SHOULD BE REVERSED BECAUSE THE EXAMINER HAS FAILED TO ESTABLISH A *PRIMA FACIE* CASE OF OBVIOUSNESS.

Claims 1-5, 7, 13, 15-19 and 22-26 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over WO 98/30706 in view of Kaufman et al., J. Clin. Invest. Vol. 89 pp. 283-292 (1992) ("Kaufman"). As will be discussed in detail below, the obviousness rejection is based on an incorrect claim interpretation and no *prima facie* case of obviousness has been established.

To establish a *prima facie* case of obviousness under 35 U.S.C. § 103, the Office must articulate a reason or rationale that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does. See, *e.g., KSR* 550 U.S. 398 (2007); *Omegaflex, Inc. v. Parker-Hannifin Corp.*, 243 Fed. App'x. 592, 595-596 (Fed. Cir. 2007) citing *KSR*. Further, the Supreme Court in *KSR* also stated that that "a court *must* ask whether the improvement is more than the predictable use of prior art elements according to their established functions." *KSR* at 1740; emphasis added.

Where the rationale used by the PTO to reject claims as obvious is based on some alleged teaching, suggestion, or motivation in the prior art that would have led one of ordinary skilled in the art to modify the prior art reference or to combine prior art reference teachings to arrive at the claimed invention, the PTO must articulate the following:

(1) a finding that there was some teaching suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings;

(2) a finding that there was a reasonable expectation of success; and

(3) whatever additional findings based on the Graham factual inquiries may be necessary in view of the facts to explain a conclusion of obviousness. See MPEP 2143(G).

It is further settled law that teaching away of prior art is a strong indication of nonobviousness. See *e.g. In re Soni*, 54 F.3d 746 (Fed. Cir. 1995). A prior art reference may be considered to teach away when a person of ordinary skill, upon reading the reference would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the Applicant. *Monarch Knitting Machinery Corp. v. Fukuhara Industrial Trading Co., Ltd.*, 139 F.3d 1009 (Fed. Cir. 1998).

According to the final Office Action dated October 8, 2008, WO 98/30706 teaches the treatment of autoimmune disorders employing a humanized IgG2b chimeric protein wherein an autoantigen peptide is inserted into the D segment of a CDR3 loop. EXHIBIT A at 7. WO 98/30706 is silent as to GAD65, GAD1 and GAD2. Kaufman, on the other hand, is cited for its disclosure that GAD65 and GAD67 proteins may be involved in Type 1 diabetes via molecular mimicry with the coxsackievirus. Kaufman does not disclose the GAD2 peptide. The Examiner states that the full length GAD65 was one of the few known IDDM autoantigens at the time of the invention and apparently on this basis concludes that it would have been obvious to insert the full length GAD65 protein into a construct of WO 98/30706 and that such a person would have had a reasonable expectation of preventing or delaying the onset of Type 1 diabetes at the priority date of the instant application. As such, the instant obviousness rejection is based on a "teaching, suggestion, or motivation" rationale—so the PTO's argument goes, since GAD65 was a known Type 1 diabetes autoantigen at the time of filing, one of skill in the art would have been motivated to insert it into the construct of WO 98/30706 and would have had a reasonable expectation of preventing or delaying the onset of Type 1 diabetes.

A. Erroneous Claim Interpretation.

The propriety of the instant obviousness rejection lies at least in part on the interpretation of the claim language. To properly interpret claim language, the Federal Circuit has held that claims must be read in view of the specification of which they are a part. *Markman v. Westview Instruments, Inc.*, 52 F.3d 967, 979 (Fed. Cir. 1995). Moreover, intrinsic evidence in the form of the patent specification should guide claim construction. Along these lines, the Federal Circuit recently reinforced the importance of the specification when interpreting claim language:

> [t]he claims, of course, do not stand alone. Rather, they are part of "a fully integrated written instrument," *Markman*, 52 F.3d at 978, consisting principally of a specification that concludes with the claims. For that reason, claims "must be read in view of the specification, of which they are a part." *Id.* at 979. As we stated in *Vitronics*, the specification "is always highly relevant to the claim construction analysis. Usually, it is dispositive; *it is the single best guide to the meaning of a disputed term.*"

Phillips v. AWH Corp., 415 F.3d 1303, 1315 (Fed. Cir. 2005) (emphasis added). Moreover, even when a term is not clearly defined in the specification, the "words of the claim must be

given their plain meaning unless the plain meaning is inconsistent with the specification." *In re Zletz*, 893 F.2d 319, 321 (Fed. Cir. 1989).

Regarding claims 1-5, 7, 13, 15-19 and 22-26, the Examiner has interpreted the phrase "at least one protein fragment or peptide inserted within the variable region; wherein (a) the protein fragment or peptide is GAD2 represented by SEQ. ID NO 4..." to read on the full length, 585 amino acid GAD65 protein being inserted within the variable region. Not only is this interpretation directly contrary to the plain meaning, it is also unreasonable in view of the teachings of the specification and knowledge of a person of ordinary skill in the art and contrary to Federal Circuit case law.

First, the plain language of the claim itself specifies that "a **protein fragment** or **peptide**" is inserted in the variable region; the claim language goes on to specify that the protein fragment or peptide is GAD2 represented by SEQ. ID No. 4. The Examiner appears to impute the open transition language "comprising" onto the "protein fragment or peptide" element thereby interpreting this element as reading on a full length protein that includes the SEQ. ID No. 4 peptide. This interpretation completely ignores the fact that the claim language specifies that "a protein fragment or peptide"—not a full length protein—is inserted in the variable region. Therefore, while the open transition language may not foreclose the possibility of the recited "protein fragment or peptide" containing some additional, unrecited amino acids, the plain language specifically calling for "a protein fragment or peptide" certainly does not read on a full length protein being inserted.

The Examiner's interpretation is also inconsistent with the specification, the entire disclosure and working examples of which describe protein fragments and peptides (but not full length proteins) inserted into variable regions. See for example page 5, lines 15 – 23, page 8, lines 1 – 11, page 9, lines 3 – 5 and 11 – 18, etc.

Furthermore, in *Genentech, Inc. v. Chiron Corp.* 112 F. 3d 495 (Fed. Cir. 1997), the court noted that "'[c]omprising' is a term of art used in claim language which means that the named elements are essential, but other elements may be added and still form a construct within the scope of the claim" (emphasis added). The Examiner's interpretation runs completely contrary to *Genentech* since it would **replace** the claim element "a protein fragment or peptide" with an entirely different claim element—a full length protein. Again, *Genentech* stands for the proposition that the recited elements—in this case "a protein fragment or peptide"—are **essential**

and thus cannot be replaced with a different element as proposed by the Examiner. While other elements may be added, no case law supports a proposition that the word "comprising" can be used to completely transform an expressly recited claim element (a protein fragment or peptide) into an altogether different element (a full length protein). In fact, *Moleculon Research Corp. v. CBS, Inc.*, 793 F.2d 1261, 1271, 229 U.S.P.Q. (BNA) 805, 812 (Fed. Cir. 1986) expressly prohibits this. The court in *Moleculon* acknowledged that "a transitional term such as 'comprising'...does not exclude additional unrecited elements, or steps (in the case of a method claim)," 793 F.2d at 1271, 229 U.S.P.Q. (BNA) at 812, but made clear that a "comprising" transition does not alter the scope of the particular claim step at issue.

> The transitional phrase, which joins the preamble of a claim with the body of a claim, is a term of art and as such affects the legal scope of a claim. While a transitional term such as "comprising" or, as in the present case, "which comprises," does not exclude additional unrecited elements, or steps (in the case of a method claim), we conclude that the transitional phrase does not, in the present case, affect the scope of the particular structure recited within the method claim's step.

For at least the foregoing reasons, the claims as properly construed do not read on the full length GAD65 protein being inserted into the variable region.

B. No rationale provided to select "a protein fragment or peptide" comprising SEQ. ID No. 4 to arrive at the claimed invention.

The Office Action provides no rationale as to why one of skill in the art would have selected SEQ. ID No. 4 from *Kaufman*'s disclosure of GAD65 and GAD67 as the protein fragment or peptide for insertion into the construct of WO98/30706. Instead, the Office Action (page 6) dated October 8, 2008 states that "[o]ne of ordinary skill in the art at the time the invention was made would have been motivated to select GAD65 as the autoantigen for use in the claimed method given the teachings of Kaufman et al. that GAD65 [the full length protein] was one of the few known IDDM autoantigens at the time of the invention." As addressed above, even if the full length GAD65 protein was one of the few known diabetic auto-antigens at the time the instant application was filed, which is not admitted and which has not been properly noticed in the record, *Kaufman* still provides no rationale to select a protein fragment or peptide comprising SEQ ID No. 4 as claimed from the virtually unlimited number of protein fragments or peptides that could be formed from the full length 585 amino acid GAD65 protein. In fact,

Kaufman is completely silent as to SEQ ID No. 4. Absent such an articulated rationale, no *prima facie* case has been established.

C. *Kaufman* teaches away.

Not only does *Kaufman* provide no motivation to the person of ordinary skill in the art to select a protein fragment or peptide comprising SEQ. ID No. 4, *Kaufman* in fact teaches away from this peptide. *Kaufman* reports results of an epitope recognition experiment to determine the ability of sera from four diabetic subjects to recognize three polypeptide segments of GAD65. Each subject was at a different stage of disease as follows: Subject 052 (high risk), Subject 723 (patient who subsequently developed IDDM); Subject 705 (at diagnosis), and Subject UC72 (advanced neuropathy). The 3 different polypeptide segments of GAD65 tested in *Kaufman* were as follows: (A) amino acids 1 – 224; (B) amino acids 224 – 398, and (C) amino acids 398 – 585. As shown in *Kaufman*'s Figure 5, inserted below for convenience, none of the four sera reacted with polypeptide (A) (which segment SEQ. ID No. 4 falls within). On the other hand, sera from two individuals reacted with both polypeptides (B) and (C) while sera from one individual reacted with only polypeptide (C).

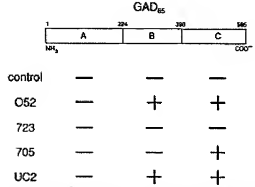

Figure 5. Epitope mapping of GAD_{65}. Three labeled segments containing the amino-terminal (*A*), middle (*B*), and carboxy-terminal (*C*) portions of GAD_{65} were immunoprecipitated with four IDDM sera that were initially characterized in the experiment shown in Fig. 4.

Since no epitopes within amino acids 1 – 224 of GAD65 were recognized by sera from IDDM patients at any stage of disease, *Kaufman*'s epitope recognition results suggest that no diabetogenic epitopes exist within this segment—the same segment in which SEQ ID No. 4 is found. Based on these results, one of skill in the art reading *Kaufman* at the time the instant invention was made would have been led away from combining a protein fragment or peptide comprising SEQ. ID No. 4 with a construct of WO 98/30706.

Because no rationale has been provided to combine Kaufman and WO 98/30706 to arrive at the claimed invention when properly construed and, in fact, *Kaufman* teaches away from such a combination, Applicants respectfully submit that no *prima facie* case of obviousness has been established. Reversal of this rejection is respectfully requested.

D. No Reasonable Expectation of Success.

The prior art can only be modified or combined to reject claims as *prima facie* obvious under a "teaching, suggestion, motivation" rationale if there is a reasonable expectation of success. MPEP 2143.02I. and *In re Merck & Co., Inc.,* 800 F.2d 1091, 231 U.S.P.Q. 375 (Fed. Cir. 1986). Even if one of skill in the art had motivation to select a protein fragment or peptide for combination with a construct of WO 98/30706, which is denied, one of skill in the art would not have had a reasonable expectation of success in preventing or delaying Type 1 diabetes. Again, because the epitope recognition studies in *Kaufman* indicated that no diabetogenic epitopes are found in amino acids 1 – 224 of GAD65, a person of ordinary skill in the art would not have had a reasonable expectation that an epitope from that region (when used in the claimed construct) would effectively prevent or delay onset of Type 1 diabetes.

For at least the foregoing reasons, no *prima facie* case of obviousness has been established and reversal of the instant obviousness rejection is respectfully requested.

3. REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH AS FAILING TO COMPLY WITH THE WRITTEN DESCRIPTION REQUIREMENT SHOULD BE REVERSED BECAUSE THE EXAMINER FAILED TO ESTABLISH A PRIMA FACIE CASE.

Claims 1-5, 7, 13, 15-19 and 22-30 stand rejected under 25 U.S.C. § 112, first paragraph as failing to comply with the written description requirement. In order to establish a *prima facie* case of lack of written description, the Examiner must show that the application as filed does not reasonably describe or convey to one of ordinary skill in the art, at the time of filing the application, that the inventor had possession of the claimed invention. MPEP § 2163.03. As stated by the Board, "the examiner has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in [the] specification disclosure a description of the invention defined by the claims. *Ex parte Sorenson*, 3 U.S.P.Q.2d 1462 (BPAI 1987). "It is not

necessary that the application describe the claim limitations exactly,...but only so clearly that persons of ordinary skill in the art will recognize from the disclosure that appellants invented processes including those limitations." *In re Wertheim*, 541 F.2d 257 (CCPA1976). Adequate description under the first paragraph of 35 U.S.C. § 112 does not require literal support for the claimed invention...Rather, it is sufficient if the originally-filed disclosure would have ***conveyed to one having ordinary skill in the art*** that an applicant had possession of the ***concept*** of what was claimed. *Ex parte Parks*, 30 U.S.P.Q.2d 1234 (BPAI 1994).

As will be discussed in detail below, no *prima facie* case of lack of written description has been established and this rejection should be reversed.

A. Rejection as to Claims 1 – 5, 7, 13, 15-19 and 22-30.

The Examiner concludes without support that the specification as filed does not provide written description for "A method comprising the administration of an immunoglobulin construct comprising a protein represented by SEQ ID NO: 4 (Claims 1 and 13)." As an initial matter, claims 1 and 13 do not specify "a protein represented by SEQ. ID. No. 4." Instead, Claim 1 specifies that the construct has "at least one protein fragment or peptide inserted within the variable region; wherein (a) the protein fragment or peptide is GAD2 represented by SEQ. ID No. 4." As such, the rejection is facially deficient and no *prima facie* case of lack of written description has been established.

Even though no *prima facie* case of lack of written description has been established, Applicants point out that claims 1 and 13 are fully supported at least at page 13, line 6; page 21, line 10; and particularly at page 45, line 20 – page 46, line 2 (the relevant text of which is reproduced below for convenience) of the specification as filed:

> Other peptides that may be inserted within the variable region within the CDR region of an Ig and utilized for creating compositions for the treatment of Type 1 diabetes as taught in the present invention are…**GAD2; corresponding to amino acid residues 206-220 of GAD 65 (SEQ. I.D. No. 4 [TYEIAPVFVLLEYVT])**; and other peptides derived from GAD65.

Applicants submit that clear support for the presently claimed invention is provided by the specification as filed and request that the instant rejection be overturned.

B. Rejection as to Claim 26.

The Examiner concludes without support that the specification as filed does not support "A method comprising the administration of an immunoglobulin construct comprising a peptide consisting of amino acid residues 206-220 of GAD65 (Claim 26). It is unclear what the basis is for this rejection. Again, the specification at page 45, line 20 to page 46, line 2 of the specification (reproduced above) makes crystal clear that a peptide consisting of amino acid residues 206-220 of GAD65 can be inserted within the CDR region of an Ig and utilized in the methods of the invention.

With respect to the Examiner's comment that "the specification does not teach a peptide consisting of amino acid residues 206-220 of *any* GAD65, e.g., mouse GAD65, rat GAD65, horse GAD65, etc.," Applicants note that the claims are to be read in light of the specification. The specification clearly defines (by chemical formula) "amino acid residues 206-220 of GAD65," as a peptide having the chemical formula set forth in SEQ. ID No. 4. Applicants again point to language at page 45, line 20 to page 46, line 2 of the specification as filed reciting: "amino acid residues 206-220 of GAD 65 (SEQ. I.D. No. 4 [TYEIAPVFVLLEYVT])." Clearly, one of skill in the art reading the specification would immediately recognize that amino acid residues 206-220 of GAD65 are referred to in the specification as SEQ. I.D. No. 4 which has the amino acid sequence TYEIAPVFVLLEYVT.

Because the originally filed disclosure would have conveyed to one having ordinary skill in the art that the Applicants had possession of the concept of what is being claimed, the instant written description rejection should be reversed.

4. REJECTION UNDER THE JUDICIALLY CREATED DOCTRINE OF OBVIOUSNESS TYPE DOUBLE PATENTING OVER CLAIMS 1 – 7 AND 13 – 16 OF U.S. 11/290,070 SHOULD BE WITHDRAWN.

Applicants respectfully note that 37 CFR § 41.37 does not require that "all pending rejections" be addressed as is stated in the Notice of Non-Compliant Appeal Brief. Rather, 37 CFR § 41.37 specifies that the appeal brief shall provide a concise statement of "each ground of rejection *presented for review*." Applicants submit that the instant double patenting rejection is a *provisional* rejection only. As is made clear by MPEP 804.2, if a provisional obviousness-type double patenting rejection between two pending applications is the only rejection remaining in

the earlier filed of the two applications, the Examiner should withdraw the rejection in the earlier filed application and permit that application to issue as a patent without a terminal disclaimer. The instant application was filed prior to U.S. 11/290,070. Therefore, assuming Applicants have overcome all other outstanding rejections in this application, the instant provisional rejection should be withdrawn as a matter of course and the instant application allowed to issue.

5. REJECTION UNDER THE JUDICIALLY CREATED DOCTRINE OF OBVIOUSNESS TYPE DOUBLE PATENTING OVER CLAIMS 1 – 7 AND 13 – 16 OF U.S. U.S. 11/425,084 SHOULD BE WITHDRAWN.

Applicants note that 37 CFR § 41.37 does not require that "all pending rejections" be addressed as is stated in the Notice of Non-Compliant Appeal Brief. Rather, 37 CFR § 41.37 specifies that the appeal brief shall provide a concise statement of "each ground of rejection *presented for review*." Applicants submit that the instant double patenting rejection is a *provisional* rejection only. As is made clear by MPEP 804.2, if a provisional obviousness-type double patenting rejection between two pending applications is the only rejection remaining in the earlier filed of the two applications, the Examiner should withdraw the rejection in the earlier filed application and permit that application to issue as a patent without a terminal disclaimer. The instant application was filed prior to U.S. 11/425,084. Therefore, assuming Applicants have overcome all other outstanding rejections in this application, the instant provisional rejection should be withdrawn as a matter of course and the instant application allowed to issue.

VIII. CONCLUSION

Appellants respectfully submit that the Examiner has failed to: (1) establish a *prima facie* case of lack of enablement or alternatively any such *prima facie* case has been rebutted; (2) establish a *prima facie* case of obviousness; and (3) establish a *prima facie* case of lack of written description. Accordingly, Appellants respectfully submit that these rejections are erroneous in law and in fact and should therefore be reversed by this Board.

The Director is authorized to charge any fees that may be required, or to credit any overpayment to Deposit Account No. 02-1818. If such a withdrawal is made, please indicate the Attorney Docket No. 3718027.00005 on the account statement.

Respectfully submitted,

K&L GATES LLP

BY _____

David B. Fournier
Reg. No. 51,696
Customer No. 24573
Phone No. 312-781-7167

Dated: January 20, 2010

CLAIMS APPENDIX

PENDING CLAIMS ON APPEAL OF U.S. PATENT APPLICATION SERIAL NO. 10/681,788

1. A method of preventing or delaying onset of Type 1 diabetes in a subject in need thereof, the method comprising administering to the subject a pharmaceutically acceptable composition comprising a soluble fusion protein, wherein the fusion protein comprises at least one immunoglobulin having a variable region comprising a CDR1, a CDR2, or a CDR3 region, the at least one immunoglobulin having at least one protein fragment or peptide inserted within the variable region; wherein (a) the protein fragment or peptide is GAD2 represented by SEQ. ID NO 4, (b) the subject has undergone insulin autoantibody seroconversion prior to said administering step and (c) the composition is administered to the subject in one or more dosage administrations.

2. The method of claim 1, wherein the immunoglobulin is human or humanized.

3. The method of claim 1, wherein the subject is a human subject.

4. The method of claim 1, wherein administration of the composition to the subject results in down regulation of an autoreactive T cell.

5. The method of claim 1, wherein the at least one protein fragment or peptide is inserted within a CDR region of the at least one immunoglobulin.

7. The method of claim 5, wherein administration of the composition to the subject results in substantially reduced activation of an autoreactive T cell specific for the at least one protein fragment or peptide.

13. The method of claim 1, wherein the at least one protein fragment or peptide consists essentially of GAD2 represented by SEQ. ID NO 4.

15. The method of claim 13, wherein the subject is GAD positive.

16. The method of claim 1, wherein the subject has not developed hyperglycemia at initiation of the administering step.

17. The method of claim 1, wherein the subject expresses a Type 1 diabetes predisposition marker at initiation of the administering step.

18. The method of claim 1, wherein upon administration of the composition to the subject, the subject undergoes a dose dependent suspension, prevention, or delay in onset of Type 1 diabetes.

19. The method of claim 1, wherein administration of a first dosage of the composition occurs before the subject has developed type-1 diabetes.

22. The method of claim 2 wherein the immunoglobulin is selected from the group consisting of IgG1, IgG2, IgG2a, IgG2b, IgG3, IgG4, IgGA, IgA1, IgA2, IgGE, IgD, IgE, or IgM.

23. The method of claim 5 wherein the at least one protein fragment or peptide is inserted within the CDR3 region of the immunoglobulin.

24. The method of claim 23 wherein the at least one protein fragment or peptide is inserted within the CDR3 region of the immunoglobulin in place of a D segment.

26. The method of claim 13 wherein the at least one protein fragment or peptide consists of amino acid residues 206-220 of GAD65.

27. The method of claim 13 wherein the subject is a human.

28. The method of claim 1 wherein the pharmaceutical composition further comprises at least one pharmaceutically acceptable carrier.

29. The method of claim 28 wherein the composition comprises an aqueous solution or suspension.

30. The method of claim 29 where the administering step is accomplished by injection or infusion.

EVIDENCE APPENDIX

EXHIBIT A: Final Office Action dated October 8, 2008.

EXHIBIT B: Pre-Appeal Brief Request for Review dated October 31, 2008.

EXHIBIT C: Notice of Panel Decision dated June 17, 2009.

EXHIBIT D: WO 98/30706. Entered in the record in OA dated 10/8/2008 at page 6.

EXHIBIT E: Kaufman. Entered in the record in OA dated 10/8/2008 at page 6.

EXHIBIT F: Marketletter. Entered in the record in OA dated 10/8/2008 at page 3.

EXHIBIT G: Anderton. Entered in the record in OA dated 10/8/2008 at page 3.

EXHIBIT H: Dong. Entered in the record in OA dated 10/8/2008 at page 3.

EXHIBIT I: Zhaghouani Declaration. Entered in the record along with the OA response dated December 19, 2007.

EXHIBIT J: Office Action Response at pages 12 – 13. Entered in the record 08/08/2008.

EXHIBIT K: Baxter and Duckworth, Drug Discovery Today: Disease Models, vol. 1, Issue 4, Dec. 2004, 451-455. Entered in the record 08/08/2008 along with Office Action Response of the same date.

RELATED PROCEEDINGS APPENDIX

None

EXHIBIT A

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/681,788	10/08/2003	Habib Zaghouani	07316.0002.CPUS01	6701

22930 7590 10/08/2008

HOWREY LLP - DC
C/O IP DOCKETING DEPARTMENT
2941 FAIRVIEW PARK DR, SUITE 200
FALLS CHURCH, VA 22042-2924

EXAMINER	
EWOLDT, GERALD R	

ART UNIT	PAPER NUMBER
1644	

MAIL DATE	DELIVERY MODE
10/08/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/681,788	ZAGHOUANI ET AL.	
	Examiner	Art Unit	
	G. R. Ewoldt, Ph.D.	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *8/11/08*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-5,7-13 and 15-30* is/are pending in the application.

4a) Of the above claim(s) *8-12,20 and 21* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-5,7,13,15-19 and 22-30* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *8/11/08*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

1. A request for continued examination (RCE) under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed 8/08/08 in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's amendment and remarks filed 8/08/08, and IDS filed 8/11/08, have been entered.

2. Claims 8-12, 20, and 21 stand withdrawn from further consideration by the examiner, 37 CFR 1.142(b), as being drawn to non-elected inventions.

Claims 1-5, 7, 13, 15-19, 22-26, and newly added Claims 27-30 are under examination.

NOTE: Claim 25 has not been withdrawn.

3. Applicant's amended Abstract has been entered.

4. In view of Applicant's amendments the previous rejection under the first paragraph of 35 U.S.C. 112 for inadequate written description has been withdrawn.

5. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

6. Claims 1-5, 7, 13, 15-19, 22-26, and newly added Claims 27-30 stand/are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Specifically, the specification provides insufficient evidence that the claimed method could effectively function as a method for preventing or delaying the onset of type I diabetes (IDDM).

As set forth previously, While the mechanism of action for the method of the instant claims is not disclosed, it appears to require inducing tolerance to GAD and altered GAD "derived" peptides. Tolerance-inducing peptide immunotherapy is well known in the immunological arts. In some cases significant results have been demonstrated in in-bred small animal models. However, said results have not been repeated in human trials. See for example, *Marketletter* (9/13/99) which teaches the complete failure in human trials of two peptides designed for tolerance induction. Both Myloral (for multiple sclerosis, MS) and Colloral (for rheumatoid arthritis, RA) provided successful results in rodent models (EAE and collagen induced arthritis, respectively).

As set forth above, the references demonstrate that even unsubstituted peptides (peptides that are not APLs) that work in *in vivo* small animal disease models cannot be expected to work in humans. Regarding the even more unpredictable APLs, Anderton (2001), teaches that:

> "This unpredictability [of APLs] led us to argue against the use of antagonist or immune deviating APL in human autoimmune disorders" (page 370).

Indeed, the reference goes on to teach that APL administration to humans can be dangerous and that in at least one case a human trial was suspended due to adverse reactions in a significant number of patients.

Other investigators have discussed additional problems in establishing human tolerance. See, for example, Dong et al. (1999):

> "Despite the fact that it has been relatively easy to induce true tolerance in small experimental animals, translating these studies into larger animals and humans has been much more difficult to achieve. Some of the hurdles that may explain this dilemma are summarized in Table 3. *Even if we have the ideal strategy to use in humans, the lack of reliable predictable assays for rejection or tolerance still does not allow us to know if a patient is truly tolerant so that immunosuppressive agents may be withdrawn*",

emphasis added.

A review of the instant specification shows just a single long example wherein a T cell response to a single insulin B chain peptide (amino acids 9-23) is inhibited in the experimental NOD mouse model of IDDM. First note that the instant claims are drawn to the use of GAD, not insulin, for the suspending, preventing or delaying the onset of IDDM. Thus, the specification offers *no* data in support of the claimed method. Interestingly, the specification discloses, that even regarding the use of an insulin peptide for the suspending, preventing or delaying the onset of IDDM, *the method of the instant claims cannot function as claimed*, emphasis added. For example, at page 28 of the specification, it is disclosed that, "Soluble Ig-INSß displayed dose dependent delay of diabetes when given at either stage [pre or post IAA conversion]. However, aggregated Ig-INSß, which induced IL-10 and TGFß-producing T cells, thus involving sustained endogenous IL-10, was protective against diabetes when given before development of insulitis *but had no effect in predisposed mice positive for IAA*", emphasis added. Further, Examples 7 and 9 teach that neither soluble nor aggregated Ig-INSß can actually prevent IDDM, but rather can only delay onset under specific conditions.

Additionally, Applicant's subsequent work demonstrates that the method of the instant claims would not be expected to function as claimed. See for example

Legge et al. (1998). Therein the authors teach that APLs function as, "T cell antagonists, partial agonists, or super agonists" (page 106). The authors go on to teach that PLP-LR stimulated PLP-1 specific T cells (paragraph spanning page 109 and 110), i.e., the T cells that would be pathogenic in an MS patient. Given that *no* experiments have been performed employing GAD peptides and derivatives thereof, it is just as likely that the method of the instant claims would actually exacerbate disease as treat or prevent it.

A set forth in *Rasmusson v. SmithKline Beecham Corp.*, 75 USPQ2d 1297, 1302 (CAFC 2005), enablement cannot be established unless one skilled in the art "would accept without question" an Applicant's statements regarding an invention, particularly in the absence of evidence regarding the effect of a claimed invention. Specifically:

> "As we have explained, we have required a greater measure of proof, and for good reason. If mere plausibility were the test for enablement under section 112, applicants could obtain patent rights to "inventions" consisting of little more than respectable guesses as to the likelihood of their success. When one of the guesses later proved true, the "inventor" would be rewarded the spoils instead of the party who demonstrated that the method actually worked. That scenario is not consistent with the statutory requirement that the inventor enable an invention rather than merely proposing an unproved hypothesis."

Thus, in view of the quantity of experimentation necessary, the lack of sufficient guidance in the specification, the lack of sufficient working examples, i.e., the specification discloses *no* data regarding the treatment or prevention of IDDM employing GAD peptides, and the unpredictability of the art, it would take undue trials and errors to practice the claimed invention.

Applicant's arguments, filed 8/08/08, have been fully considered but are not found persuasive. Applicant argues that the utility rejection under 35 U.S.C., first paragraph should be withdrawn.

Applicant is advised that there is no utility rejection outstanding (35 U.S.C. 101). The instant rejection is for lack of enablement under the first paragraph of 35 U.S.C. 112.

At page 12 of the Remarks Applicant argues that data derived employing a NOD mouse model should be accepted, again citing the Inventor's 12/19/07 1.132 declaration.

The Inventor's declaration was addressed previously. Regarding the Inventor's 1.132 declaration, it appears that the Inventor has recently established some efficacy in the NOD mouse model employing an unidentified "soluble Ig-GAD2". The Inventor's results are noted, however, as set forth above, said results are not enabling for the use of the claimed method for the preventing or delaying the onset of IDDM in humans. Also, the work was apparently done after the effective filing date and

therefore cannot be relied upon to show enablement at the time of filing as is required.

Applicant argues that Couzin (2003), "does not evidence failure of the NOD model to predict success in humans" and, "No indication is found in Couzin that the researchers were in fact attempting to induce "tolerance to insulin.""

Regarding the induction of tolerance in mouse models, such is well-known to immunologists. That tolerance induced in mouse models has not translated to efficacy in humans is also well-known to the ordinarily skilled immunologist. Again, see the Abstract in Harrison (2008, of record). Regarding the DPT-1 trial referenced in Couzin, again it is well-known to the ordinarily skilled immunologist that the trial was indeed a failed attempt to induce tolerance in humans. For Applicant's edification a review of the results, including a description of the attempt to induce immune tolerance, is enclosed (see Skylar et al. (2005)).

Applicant cites Harrison (2008, of record) as "proof of concept".

A 2008 "proof of concept" cannot be considered enabling for the 2002 invention of the instant claims. Indeed, a "proof of concept" is evidence that an invention does not yet exist as a "concept" is not an "invention". Additionally, it is an Applicant's obligation to supply an enabling disclosure without reliance on what others *may* publish after he has filed an application on what is supposed to be a completed invention. If he cannot supply enabling information, he is not yet in a position to file.

Applicant argues the separateness of the rejections for lack of enablement and obviousness, arguing that Applicant's have enabled the claimed invention. Applicant argues that the invention was unpredictable prior to the Applicant's invention. Interestingly, in the remarks regarding the obviousness rejection, Applicant argues a lack of expectation of success.

Applicant's arguments are noted but the claimed invention is not now enabled nor was it enabled at the time of filing. A review of the instant specification reveals *no* data employing the construct used in the claimed method. It is unclear then how Applicant can argue that the invention is both enabled for it's full scope, but at the same time unexpected. The

Examiner's more tenable position is that, while the invention might delay onset of diabetes in some experimental mice (and can thus be found to be obvious), it is not enabled for the preventing of disease in any species nor the preventing or delay of disease in humans.

7. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

8. Claims 1, 2, 4, 5, 7, 13, 15-19, 22-24, and newly added Claims 28-30 stand/are rejected under 35 U.S.C. 103(a) as being unpatentable over WO 98/30706 in view of Kaufman et al. (1992).

As set forth previously, WO 98/30706 teaches the treatment of autoimmune disorders, including IDDM, (see particularly pages 10 and 19) employing an engineered fusion protein, e.g., a humanized IgG_{2b} chimeric protein wherein an autoantigen peptide is inserted into the D segment of a CDR3 loop (see particularly Figure 1, page 13, and Example II).

The method differs from the claimed invention only in that it does not teach the use of GAD65 as the autoantigen employed for the treatment of IDDM.

Kaufman et al. teach that GAD65 (which would comprise amino acid residues 206-220 and 524-543), along with insulin, is a well-known IDDM autoantigen (see particularly page 283, column 2).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to perform the method of WO 98/30706 for the treatment of IDDM employing the autoantigen of Kaufman et al. One of ordinary skill in the art at the time the invention was made would have been motivated to select GAD65 as the autoantigen for use in the claimed method given the teachings of Kaufman et al. that GAD65 was one of the few known IDDM autoantigens at the time of the invention. Regarding the timing of administration of the Ig-fusion protein set forth in claims such as 3, 16, 17, etc., said timing would comprise only routine optimization which would fall well within the purview of one of skill in the art at the time of the invention.

Applicant's arguments, filed 8/08/08, have been fully considered but are not found persuasive. Applicant argues a lack of asserted obviousness.

A review of the primary reference reveals that it is by the Inventor wherein he teaches that an identical immunoglobulin construct, save for the choice of inserted antigen, can be employed to treat diabetes. Thus, inserting one of the most well-known and well-characterized diabetes antigens into Applicant's own known immunoglobulin construct, and then using said construct to delay diabetes, would have been obvious.

Applicant argues no reasonable expectation of success. As set forth above, there is a reasonable expectation that the construct of the claims could be used to delay diabetes onset in experimental animals. Also note that the reference is Applicant's own work and Applicant says that the immunoglobulin construct can be used to treat diabetes. To now argue lack of expectation of success when faced with an obviousness rejection is not persuasive. Note that Claim 26 reciting a fragment consisting of specific residues of GAD65 is not included in the rejection because there is no teaching of employing only a specific 14 amino acid fragment. All other claims, however, encompass the use of full length GAD65 which is obvious.

Applicant argues that not all claim elements are taught by the references, specifically administration of the immunoglobulin construct after insulin autoantibody seroconversion.

Applicant is advised that the claimed method would be obvious for delaying diabetes at any stage in which it might still be delayed.

9. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be

commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

10. Claims 1-5, 7, 13, 15-19, 22-25, and 27-30 stand/are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claims 1-7 and 13-16 of U.S. Patent Application No. 11/290,070. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '070 application recite a method comprising treating IDDM with a GAD construct such as would be encompassed by that recited in Claim 1.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

11. Claims 1-5, 7, 13, 15-19, 22-25, and 27-30 stand/are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claims 1-7 and 13-16 of U.S. Patent Application No. 11/425,084. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '084 application recite a method comprising treating IDDM with a GAD construct such as would be encompassed by that recited in Claim 1.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Applicant defers a response regarding the remaining rejections until the finding of allowable claims.

12. The following are new grounds for rejection necessitated by Applicant's amendment.

13. Claims 1-5, 7, 13, 15-19, and 22-30 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art

that the inventor(s), at the time the application was filed, had possession of the claimed invention. This is a new matter written description rejection.

The specification and the claims as originally filed do not provide support for the invention as now claimed, specifically:

A) A method comprising the administration of an immunoglobulin construct comprising a protein represented by SEQ ID NO:4 (Claims 1 and 13).

B) A method comprising the administration of an immunoglobulin construct comprising a peptide consisting of amino acid residues 206-220 of GAD65 (Claim 26).

Applicant cites pages 13, 21, 45, and 26 in support of the claimed method.

A review of the specification reveals that the peptide of SEQ ID NO:4 is found at page 46 of the specification. The specification, however, does not teach the peptides as part of an immunoglobulin construct. Further, the specification does not teach a peptide consisting of amino acid residues 206-220 of *any* GAD65, e.g., mouse GAD65, rat GAD65, horse GAD65, etc.

14. No claim is allowed.

15. All claims are drawn to the same invention claimed in the application prior to the entry of the submission under 37 CFR 1.114 and could have been finally rejected on the grounds and art of record in the next Office action if they had been entered in the application prior to entry under 37 CFR 1.114. Accordingly, **THIS ACTION IS MADE FINAL** even though it is a first action after the filing of a request for continued examination and the submission under 37 CFR 1.114. See MPEP § 706.07(b). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event,

however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

16. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Dr. Gerald Ewoldt whose telephone number is (571) 272-0843. The examiner can normally be reached Monday through Thursday from 7:30 am to 5:30 pm. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Eileen O'Hara, Ph.D. can be reached on (571) 272-0878.

17. **Please Note:** Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197

/G.R. Ewoldt/
G.R. Ewoldt, Ph.D.
Primary Examiner
Technology Center 1600

EXHIBIT B

Doc Code: AP.PRE.REQ

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PRE-APPEAL BRIEF REQUEST FOR REVIEW

Docket Number (Optional)	
119742-005	

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to "Mail Stop AF, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450" [37 CFR 1.8(a)]

on _____

Signature _____

Typed or printed name _____

Application Number	Filed
10/681,788	October 8, 2003
First Named Inventor	
Habib Zaghouani, et al.	
Art Unit	**Examiner**
1644	Gerald R. Ewoldt

Applicant requests review of the final rejection in the above-identified application. No amendments are being filed with this request.

This request is being filed with a notice of appeal.

The review is requested for the reason(s) stated on the attached sheet(s).
Note: No more than five (5) pages may be provided.

I am the

- ☐ applicant/inventor.
- ☐ assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96)
- ☑ attorney or agent of record. Registration number 51,696
- ☐ attorney or agent acting under 37 CFR 1.34. Registration number if acting under 37 CFR 1.34 _____

Signature

David B. Fournier
Typed or printed name

312.781.7167
Telephone number

October 30, 2008
Date

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☐ *Total of _____ forms are submitted.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Habib Zaghouani
Appl. No.:	10/681,788
Conf. No.:	6701
Filed:	October 8, 2003
Title:	SUSTAINED TREATMENT OF TYPE 1 DIABETES AFTER EXPRESSION OF PREDISPOSITION MARKERS
Art Unit:	1644
Examiner:	Edwoldt, G.R.
Docket No.:	0119742-005

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PRE-APPEAL BRIEF REQUEST FOR REVIEW

This paper is submitted in response to the final Office Action dated October 8, 2008.

I. **Rejection under 35 U.S.C. § 112, first paragraph.**

Claims 1-5, 7, 13, 15-19 and 22-30 stand rejected under 35 U.S.C. § 112, first paragraph on the alleged basis that there is "insufficient evidence that the claimed method could effectively function as a method for preventing or delaying the onset of type 1 diabetes (IDDM)." Office Action ("OA") dated 10/08/2008 at p. 3. This rejection omits one or more essential elements needed for a *prima facie* rejection; additionally, clear factual errors exist.

As a preliminary matter, it has been unclear during prosecution whether the instant § 112, first paragraph rejection is on the basis that the asserted utility is not credible (MPEP 2107.01IV), or under the "how to use" requirement of that paragraph. If the rejection is indeed based on an alleged lack of credible utility under §112, Applicants have previously established clear error as well as omission of essential elements required for such a *prima facie* rejection. See OA Response dated August 7, 2008, Section II (Page 9 – 15).

If the instant rejection, instead, relates to the "how to use" requirement of §112, first paragraph, clear factual errors as well as omissions in the *prima facie* rejection exist as are discussed below.

A. **Omission of essential element needed for *prima facie* rejection.**

It is settled law that a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented ***must*** be taken as in compliance with the enablement requirement of the first paragraph of § 112 ***unless*** there is reason to doubt the objective truth of the statements contained therein. MPEP 2164.04 citing *In re Marzocchi*, 58

C.C.P.A. 1069, 439 F.2d 220, 223, 169 U.S.P.Q. (BNA) 367, 369 (CCPA 1971). Thus, the PTO has the initial burden of challenging a ***presumptively correct*** assertion of utility in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would ***reasonably doubt*** the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. *See In re Bundy*, 642 F.2d 430, 433, 209 U.S.P.Q. (BNA) 48, 51 (CCPA 1981).

The PTO has simply not provided any evidence showing that one of ordinary skill in the art would ***reasonably doubt*** the asserted utility of the claimed invention and has therefore not met its initial burden. The only evidence provided by the PTO for lack of enablement shows that, using ***fundamentally different*** therapeutic agents than those claimed, tested in diseases ***other than*** Type 1 diabetes ("TID") as presently claimed, some researches have achieved tolerance results in animal models that have been difficult to reproduce in humans. See OA dated 10/08/2008 at page 3. Specifically, *Marketletter* deals with two unsubstituted peptides (much different than the claimed Ig-GAD2 fusion protein) tested in Multiple Sclerosis and Rheumatoid Arthritis, not TID. *Anderton* is a review article generally discussing administration of native antigen and analogue peptides in a Multiple Sclerosis model which, again, is a much different therapeutic modality than the claimed fusion protein and a different disease state than TID. Finally, *Dong* is merely a general review of tissue graft transplant tolerance (not to treatment of any autoimmune disorder let alone TID) and contains nothing to call in to question use of the ***presently claimed*** fusion protein construct for delaying or preventing TID. At most, these references are only tangentially related to the presently claimed invention in that they relate in various ways to tolerance. However, they simply do not cast any doubt, let alone any reasonable doubt, on the use of the ***presently clamed invention*** which entails an altogether different therapeutic agent and different disease state than those discussed in the cited references. Applicants respectfully submit that the burden of challenging the presumptively correct assertion of the manner of using the invention has not been met. Withdrawal of this rejection is therefore respectfully requested.

B. Clear Legal Error.

1. Post-filing date evidence *must* be considered and *can* be used to demonstrate enablement at the time of filing.

To further demonstrate that Applicants' claimed invention was enabled at the time of filing, Applicants submitted a declaration under 37 CFR 1.312 showing that the claimed method prevents and/or delays the onset of TID in the gold standard NOD mouse model for that disease. See Declaration of Dr. Habib Zaghouani ("Zaghouani Declaration") made of record December 19, 2007. The OA states that "the work [summarized in the Declaration] was apparently done after the effective filing date and therefore **cannot be relied upon to show enablement at the time of**

filing as is required." OA dated 10/8/2008, page 4, last paragraph – page 5, first paragraph (emphasis added). This statement is clearly erroneous. Contrary to the Examiner's position, both the MPEP and Federal Circuit case law are clear that post-filing date declarations can be used to demonstrate that the claimed invention was enabled when filed and **must be considered** when submitted. MPEP 2164.05 and *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995).

The *Brana* case is particularly on point. In *Brana*, the Federal Circuit stated in the context of a 112, first paragraph enablement rejection that "[e]ven if one skilled in the art would have reasonably questioned the asserted utility, *i.e.*, even if the PTO met its initial burden thereby shifting the burden to the applicants to offer rebuttal evidence, applicants proffered sufficient evidence to convince one of skill in the art of the asserted utility." *Id* at 1567. In *Brana*, the applicants provided a post-filing date declaration showing that compounds within the scope of the claims exhibited significant anti-tumor activity against the L1210 standard tumor model *in vivo*. According to the court, such evidence alone should have been sufficient to satisfy applicants' burden. The court noted that "[t]he Kluge declaration, though dated ***after*** applicant's filing date, could be used to substantiate any doubts as to the asserted utility since this pertains to the accuracy of a statement already in the specification. It does not render an insufficient disclosure enabling, **but instead goes to prove that the disclosure was in fact enabling when filed (i.e., demonstrated utility)**." (Emphasis added, internal citation omitted).

Like the declaration submitted in *Brana*, the Zaghouani Declaration submitted in the instant case also pertains to the accuracy of statements already in the specification and evidences that the original disclosure was in fact enabling when filed. Specifically, the Zaghouani Declaration illustrates the accuracy of disclosure in the specification (e.g. pages 45-46 and elsewhere) that the claimed Ig-GAD2 construct is useful for preventing and/or delaying onset of TID.

It was clear legal error for the Examiner to disregard the Zaghouani Declaration and to take the position that post-filing data submitted via declaration cannot be relied on to show enablement at the time of filing of the application. Withdrawal of this rejection is respectfully requested.

2. No requirement for human data exists.

The Examiner further takes the position that "while the invention might delay onset of diabetes in some experimental mice…it is not enabled for the prevention of disease in any species nor the preventing or delay of disease ***in humans***." OA dated 10/08/2008 at page 4 and 6. The Examiner attempts to support this conclusion by citing to instances in which treatment (albeit with a fundamentally different type of therapeutic agent and in different diseases than TID as discussed above) succeeded in animals but failed in humans—for example *Marketletter.* That position—the same position taken by the PTO and summarily rejected by the Federal Circuit in *Brana*—is also clear legal error.

In *Brana*, the PTO argued in the context of a 112, first paragraph rejection that *in vivo* test results in animals are not reasonably predictive of the success of the claimed compounds for treating cancer in humans. *Id* at 20. In response, the Federal Circuit stated:

> The Commissioner, as did the Board, confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption... proof of an alleged pharmaceutical property for a compound by statistically significant tests with standard experimental animals is sufficient to establish utility. *In re Krimmel*, 48 C.C.P.A. 1116, 292 F.2d 948, 953...In concluding that similar *in vivo* tests were adequate proof of utility the court in *In re Krimmel* stated: We hold as we do because it is our firm conviction that one who has taught the public that a compound exhibits some desirable pharmaceutical property **in a standard experimental animal has made a significant and useful contribution to the art, even though it may eventually appear that the compound is without value in the treatment of humans**. *Id*. At 22. (emphasis added)

As has been clearly established in the record, the NOD mouse model used in the experiment described in the Zaghouani Declaration is considered the gold standard animal model for TID. See OA Response dated 08/07/2008, pages 12 – 13. It was clear error to reject the instant claims under 35 U.S.C. § 112, first paragraph on the alleged basis that successful results in the gold standard animal model for TID do not necessarily translate to humans. Withdrawal of this rejection is respectfully requested.

II. Rejection Under 35 U.S.C. 103(a).

Claims 1, 2, 4, 5, 7, 13, 15-19, 22-24 and 28-30 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over WO 98/30706 in view of Kaufman et al., J. Clin. Invest. Vol. 89 pp. 283-292 (1992) ("Kaufman"). Applicants respectfully submit that clear factual errors exist in this rejection and essential elements are missing from the *prima facie* case.

A. Clear Factual Errors.

The Examiner states that "[a]ll other claims [besides claim 26], however, encompass the use of **full length GAD65** which is obvious." OA at p. 7, 2nd paragraph (emphasis added). This statement is factually incorrect. Claim 1 as amended in the OA response dated 08/07/2008 (page 5) specifies that "the protein fragment or peptide **is GAD2 represented by SEQ. ID NO 4.**" (emphasis added). As is disclosed at page 45 of the instant specification, GAD2 is a 15 amino acid sequence corresponding to residues 206-220 of GAD65 and is represented as SEQ. ID NO 4. All additional pending claims depend from claim 1. The claims do **not** specify that the protein fragment or peptide is full length GAD65 as asserted. The instant obviousness rejection was made based on a factually incorrect reading of the claim language. Withdrawal of this rejection is respectfully requested.

B. Essential elements missing from *prima facie* case.

The OA states that "...claim 26 reciting a fragment consisting of specific residues of GAD 65 is not included in the rejection because there is no teaching of employing a specific 14 (sic) amino acid fragment such as GAD2 represented by SEQ. ID NO 4. All other claims, however, encompass the use of full length GAD65 which is obvious." OA dated 10/08/2008 at page 7, 2nd paragraph. As pointed out above, **all** of the pending claims in fact specify that the "protein fragment or peptide is GAD2 represented by SEQ. ID NO 4," not the full length GAD65 protein. As such, no *prima facie* case has been established with respect to any claims just as the Office stated it had not been established with respect to claim 26. The OA response dated 08/07/2008 at pages 15 – 19 further sets forth the absence of essential elements needed for a *prima facie* case of obviousness of the presently pending claims when properly construed.

III. Rejection Under 35 U.S.C. 112, first paragraph.

Claims 1-5, 7, 13, 15-19 and 22-30 stand rejected under 35 U.S.C. 112, first paragraph as allegedly introducing new matter. This rejection contains clear factual error. The OA states that "[t]he specification does not teach the peptides as part of an immunoglobulin construct." This is clearly factually incorrect. GAD2 represented by SEQ. ID No. 4 is the only peptide presently claimed. Applicant draws the PTO's attention to page 45, line 20 – page 46, line 2 and reproduces that text for convenience:

> Other peptides that may be inserted within the variable region **within the CDR region of an Ig and utilized for creating compositions for the treatment of type 1 diabetes as taught in the present invention** are: GAD1 (Glutamic acid decarboxylase-65 also known as "GAD65"); corresponding to amino acid residues 524-543 of GAD 65 (Seq. I.D. No. 3 [SRLSKVAPVIKARMMEYGTT]) to create chimera Ig-GAD1; and 2) **GAD2; corresponding to amino acid residues 206-220 of GAD 65 (Seq. I.D. No. 4 [TYEIAPVFVLLEYVT])**; and other peptides derived from GAD65.

Clearly the specification teaches GAD2 as part of an immunoglobulin construct for use in the instant invention. Withdrawal of this rejection is therefore respectfully requested.

Conclusion

The application is believed to be in condition for allowance. Early and favorable considerations is respectfully requested.

Respectfully submitted,

BELL, BOYD & LLOYD LLP

BY _____
David B. Fournier
Reg. No. 51,696
October 30, 2008

EXHIBIT C

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/681,788	10/08/2003	Habib Zaghouani	119742-005	6701

24573 7590 06/17/2009

K&L Gates LLP
P.O. Box 1135
CHICAGO, IL 60690

EXAMINER	
EWOLDT, GERALD R	
ART UNIT	PAPER NUMBER
1644	
MAIL DATE	DELIVERY MODE
06/17/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of Panel Decision from Pre-Appeal Brief Review	Application/Control No.	Applicant(s)/Patent under Reexamination	
	10/681,788	ZAGHOUANI ET AL.	
	RAM R. SHUKLA	Art Unit 1644	

This is in response to the Pre-Appeal Brief Request for Review filed 31 October 2008.

1. ☐ **Improper Request** – The Request is improper and a conference will not be held for the following reason(s):

☐ The Notice of Appeal has not been filed concurrent with the Pre-Appeal Brief Request.
☐ The request does not include reasons why a review is appropriate.
☐ A proposed amendment is included with the Pre-Appeal Brief request.
☐ Other: .

The time period for filing a response continues to run from the receipt date of the Notice of Appeal or from the mail date of the last Office communication, if no Notice of Appeal has been received.

2. ☒ **Proceed to Board of Patent Appeals and Interferences** – A Pre-Appeal Brief conference has been held. The application remains under appeal because there is at least one actual issue for appeal. Applicant is required to submit an appeal brief in accordance with 37 CFR 41.37. The time period for filing an appeal brief will be reset to be one month from mailing this decision, or the balance of the two-month time period running from the receipt of the notice of appeal, whichever is greater. Further, the time period for filing of the appeal brief is extendible under 37 CFR 1.136 based upon the mail date of this decision or the receipt date of the notice of appeal, as applicable.

☒ The panel has determined the status of the claim(s) is as follows:
Claim(s) allowed: *None*.
Claim(s) objected to: *None*.
Claim(s) rejected: *1-5, 7, 13, 15-19, 22-30*.
Claim(s) withdrawn from consideration: *8-12, 20, 21*.

3. ☐ **Allowable application** – A conference has been held. The rejection is withdrawn and a Notice of Allowance will be mailed. Prosecution on the merits remains closed. No further action is required by applicant at this time.

4. ☐ **Reopen Prosecution** – A conference has been held. The rejection is withdrawn and a new Office action will be mailed. No further action is required by applicant at this time.

All participants:

(1) */RAM R. SHUKLA/*. (3)*Gerald Ewoldt*.

(2) *Robert Wax*. (4)_____.

EXHIBIT D

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/62, A61K 39/385, C07K 19/00, C12N 5/10

A1

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(30) Priority Data:
08/779,767 7 January 1997 (07.01.97) US

(71) Applicant *(for all designated States except US)*: ALLIANCE PHARMACEUTICAL CORP. [US/US]; 3040 Science Park Road, San Diego, CA 92121 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: ZAGHOUANI, Habib [TN/US]; 1408 Knightsbridge Drive, Knoxville, TN (US).

(74) Agent: ALTMAN, Daniel, E.; Knobbe, Martens, Olson and Bear, LLP, 16th floor, 620 Newport Center Drive, Newport Beach, CA 92660 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: COMPOUNDS, COMPOSITIONS AND METHODS FOR THE ENDOCYTIC PRESENTATION OF IMMUNOSUPPRESSIVE FACTORS

(57) Abstract

Immunomodulating agents comprising at least one Fc receptor ligand and at least one immunosuppressive factor are provided as are methods for their manufacture and use. The immunomodulating agents may be in the form of polypeptides or chimeric antibodies and preferably incorporate an immunosuppressive factor comprising a T cell receptor antagonist. The compounds and compositions of the invention may be used to selectively suppress the immune system to treat symptoms associated with immune disorders such as allergies, transplanted tissue rejection and autoimmune disorders including lupus, rheumatoid arthritis and multiple sclerosis.

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COMPOUNDS, COMPOSITIONS AND METHODS FOR THE ENDOCYTIC PRESENTATION OF IMMUNOSUPPRESSIVE FACTORS

Field of the Invention

The present invention generally relates to compounds, compositions and methods for the effective endocytic presentation of immunosuppressive factors. More particularly, the present invention is directed to compounds, methods and compositions comprising immunosuppressive factors that are useful for the treatment of various disorders including, but not limited to, autoimmune disorders. In preferred embodiments the immunosuppressive factors may be T cell receptor antagonists or agonists. Other embodiments of the invention provide for the induction of tolerance in neonates or infants.

Background of the Invention

Vertebrates possess the ability to mount an immune response as a defense against pathogens from the environment as well as against aberrant cells, such as tumor cells, which develop internally. The immune response is the result of complex interactions between a variety of cells and factors, but generally comprises two main facets. One is a cellular component, in which specialized cells directly attack an offending agent (bearing an antigen) while the other is a humoral component, in which antibody molecules bind specifically to the antigen and aid in its elimination. Acting in concert, the individual elements are quite effective in limiting the initial onslaught of invading pathogens and eliminating them from the host.

The primary cells involved in providing an immune response are lymphocytes which generally comprise two principal classes. The first of these, designated B cells or B lymphocytes, are typically generated in bone marrow and are, among other duties, responsible for producing and secreting antibodies. B cell antibody products tend to react directly with foreign antigens and neutralize them or activate other components of the immune systems which then eliminate them. In particular, opsonizing antibodies bind to extracellular foreign agents thereby rendering them susceptible to phagocytosis and subsequent intracellular killing. On the other hand T cells or T lymphocytes, which generally develop or mature in the thymus, are responsible for mediating the cellular immune response. These cells do not recognize whole antigens but, instead, respond to short peptide fragments thereof bound to specialized proteins which appear on the surface of the surface of a target cell. More particularly, it appears that proteins produced within the cell, or taken up by the cell from the extracellular milieu, are continually degraded to peptides by normal metabolic pathways. The resulting short fragments associate with intracellular major histocompatibility complex (MHC) molecules and the MHC-peptide complexes are transported to the surface of the cell for recognition by T cells. Thus, the cellular immune system is constantly monitoring a full spectrum of proteins produced or ingested by the cells and is posed to eliminate any cells presenting foreign antigens or tumor antigens; i.e. virus infected cells or cancer cells.

The general structure of immunoglobulin G (IgG), the most common of mammalian antibodies, is shown schematically in Figure 1. As illustrated, IgG is a tetrameric protein complex comprising two identical heavy (H)

chains and two identical immunoglobulin light (L) chains. These chains are joined together by disulfide bonds to form the Y-shaped antibody complex. In solution however, the molecule takes on a more globular shape and readily bind to foreign antigens present in biological fluids.

Amino acid sequence analysis of immunoglobulins has led to the definition of specific regions with various functional activities within the chains. Each light chain and each heavy chain has a variable region (V_L and V_H respectively) defined within the first 110 amino terminal residues. Three dimensional pairing of the V_L and V_H regions constitute the antigen-recognition portion or "antigen combining site" ("ACS") of immunoglobulin molecule. Because of the tetrameric nature of immunoglobulins, there are two identical antigen combining sites per molecule. The variable domains of these chains are highly heterogeneous in sequence and provide the diversity for antigen combining sites to be highly specific for a large variety of antigenic structures. The heterogeneity of the variable domains is not evenly distributed throughout the variable regions, but is located in three segments, called complementarity determining regions ("CDRs") designated CDR 1, CDR 2 and CDR 3. For further information regarding these structures see Watson et al., 1987, *Molecular Biology of the Gene*, Fourth Edition, Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA incorporated herein by reference.

Each of the heavy chains also includes a constant region defining a particular isotype and assigns the immunoglobulin to one of the immunoglobulin classes and subclasses. The constant region contains units called domains (i.e. C_{H1}, C_{H2}, etc.) which do not vary significantly among antibodies of a single class. The constant region does not participate in antigen binding, but can be associated with a number of biological activities known as "effector functions", such as binding to Fc receptors on cell surfaces of antigen presenting cells (APC's) as well as binding to complement proteins. Antigen presenting cells such as dendritic cells and macrophages are, among other features, generally distinguished by the presence of an Fc receptor. Consequently, if an antibody is bound to a pathogen, it can then link to a phagocyte via the Fc portion. This allows the pathogen to be ingested and destroyed by the phagocyte, a process known as opsonization. Moreover, as will be discussed in more detail below, various pathogenic antigens may be processed and displayed by the APC to further stimulate an immune response.

Unlike the heavy chains, the light chains have a single constant domain (C_L). A light chain pairs with a heavy chain through a disulfide bond which attaches heavy constant region C_{H1} to C_L. In addition, the heavy chains have a hinge region separating constant regions C_{H1} and C_{H2} from the remainder of the molecule. It is this hinge region that is largely responsible for the flexibility of the tetramer. The two heavy chains of the molecule pair together through disulfide bonds at the junction between the hinge region and C_{H2}.

In order to provide such an extensive repertoire, immunoglobulin genes have evolved so as permit the production of vast numbers of different immunoglobulin proteins from a finite number of genes i.e. inherent polymorphism. Due to inherent polymorphism, mammals are able to produce antibodies to a seemingly infinite variety of antigens. For a review of immunoglobulin genetics and protein structure see Lewin, "Genes III", John Wiley and Sons, N.Y. (1987) and Benjamini and Leskowitz, 1988, Immunology, Alan R. Liss, Inc., New York which is incorporated herein by reference.

In the past few years antibodies have become extremely important in diagnostic and therapeutic applications due to their diversity and specificity. Increasingly, molecular biology techniques have been used to expand the variety and availability of antibodies for scientific applications. For instance, a single antibody producing B cell can be immortalized by fusion with a tumor cell and expanded to provide an *in vitro* source of antibodies of a single specificity known as a "monoclonal antibody" (mAb). Such an immortal B cell line is termed a "hybridoma."

Until recently, the source of most mAb has been murine (mouse) hybridomas cultured *in vitro*. That is, a mouse was typically injected with a selected antigen or immunogen. Subsequently, the animal was sacrificed and cells removed from its spleen were fused with immortal myeloma cells. Although they have been used extensively in diagnostic procedures, murine mAb have not proven to be well suited for therapeutic applications in most mammals including humans. In part, this is due to the fact that murine antibodies are recognized as foreign by other mammalian species and elicit an immune response which may itself cause illness or undesirable side effects.

To overcome at least some of the problems of immune responses generated by foreign mAb and the lack of suitable human mAb, genetic engineering has been used to construct humanized chimeric immunoglobulin molecules which contain the antigen binding complementarity determining regions of the murine antibodies but in which the remainder of the molecule is composed of human antibody sequences which are not recognized as foreign. Such antibodies have been used to treat tumors as the mouse variable region recognizes the tumor antigen and the humanized portion of the molecule is able to mediate an immune response without being rapidly eliminated by the body. See, for example, Jones et al., *Nature*, 321:522-525 (1986) which is incorporated herein by reference.

Other uses of such antibodies are detailed in co-pending U.S.S.N. 08/383,276 and PCT Publication No. WO 94/14847 which are also incorporated herein by reference. In these cases epitopes of foreign antigens such as viral or bacterial epitopes are grafted onto the hypervariable region of an immunoglobulin to induce a response. That is, the engineered antibodies are used as a vaccine to provoke an immune response and confer long term immunogenic memory thereby allowing the subject to fight off subsequent infections.

These and more traditional vaccines are effective in that they stimulate both prongs of the immune system. Despite the intricacies associated with the humoral component of the immune response, it would not, in and of itself, be capable of effectively protecting an animal from the myriad pathogenic assaults to which it is subject each day. Rather, it is only the presence of a highly evolved cellular response that allows higher organisms to survive and proliferate.

As indicated above, T lymphocytes or T cells, which arise from precursors in the bone marrow, are central players in the immune response against invading viruses and other microbes. The progenitor stem cells migrate to the thymus where, as so-called thymocytes, they become specialized. In particular, they begin to display the receptor molecules that later enable mature T cells to detect infection. To be beneficial, T cells must be able to attach through their receptors to microbial antigens (protein markers signaling an invader's presence). At the same time, they should be blind to substances made by the body as self-reactive T cells can destroy normal tissues. Typically, only those thymocytes that make useful receptors will mature fully and enter the bloodstream to patrol the body.

Others that would be ineffectual or would attack the body's own tissue are, in healthy individuals, eliminated through apoptosis prior to leaving the thymus.

Mature T cells that finally enter the circulation, either as cytolytic T lymphocytes or T helper cells, remain at rest unless they encounter antigens that their receptors can recognize. Upon encountering the specific antigens for which the lymphocytes have affinity, they proliferate and perform effector functions, the result of which is elimination of the foreign antigens.

T cells have been classified into several subpopulations based on the different tasks they perform. These subpopulations include helper T cells (T_h), which are required for promoting or enhancing T and B cell responses; cytotoxic (or cytolytic) T lymphocytes (CTL), which directly kill their target cells by cell lysis; and suppressor T cells (T_s) which down-regulate the immune response. In each case the T cells recognize antigens, but only when presented on the surface of a cell by a specialized protein complex attached to the surface of antigen presenting cells. More particularly, T cells use a specific receptor, termed the T cell antigen receptor (TCR), which is a transmembrane protein complex capable of recognizing an antigen in association with the group of proteins collectively termed the major histocompatibility complex (MHC). Thousands of identical TCR's are expressed on each cell. The TCR is related, both in function and structure, to the surface antibody (non-secreted) which B cells use as their antigen receptors. Further, different subpopulations of T cells also express a variety of cell surface proteins, some of which are termed "marker proteins" because they are characteristic of particular subpopulations. For example, most T_h cells express the cell surface CD4 protein, whereas most CTL and T_s cells express the cell surface CD8 protein. These surface proteins are important in the initiation and maintenance of immune responses which depend on the recognition of, and interactions between, particular proteins or protein complexes on the surface of APCs.

For some time it has been known that the major histocompatibility complex or MHC actually comprises a series of glycosylated proteins comprising distinct quaternary structures. Generally, the structures are of two types: class I MHC which displays peptides from proteins made inside the cell (such as proteins produced subsequent to viral replication), and class II MHC, which generally displays peptides from proteins that have entered the cell from the outside (soluble antigens such as bacterial toxins). Recognition of various antigens is assured by inherited polymorphism which continuously provides a diverse pool of MHC molecules capable of binding any microbial peptides that may arise. Essentially, all nucleated cells produce and express class I MHC which may exhibit naturally occurring peptides, tumor associated peptides or peptides produced by a viral invader. Conversely, only a few specialized lymphoid cells, those generally known as antigen presenting cells, produce and express class II MHC proteins. Regardless of the cell type, both classes of MHC carry peptides to the cell surface and present them to resting T lymphocytes. Ordinarily T_h cells recognize class II MHC-antigen complexes while CTL's tend to recognize class I MHC-antigen complexes.

When a resting T cell bearing the appropriate TCR encounters the APC displaying the peptide on its surface, the TCR binds to the peptide-MHC complex. More particularly, hundreds of TCR's bind to numerous peptide-MHC complexes. When enough TCRs are contacted, the cumulative effect activates the T cell. Receptors on T cells that are responsible for the specific recognition of, and response to, the MHC-antigen complex are composed of a complex

of several integral plasma membrane proteins. As with the MHC complex previously discussed, a diverse pool of TCR's is assured by inherent polymorphism leading to somatic rearrangement. It should be emphasized that, while the pool of TCR's may be diverse, each individual T cell only expresses a single specific TCR. However, each T cell typically exhibits thousands of copies of this receptor, specific for only one peptide, on the surface of each cell. In addition, several other types of membrane associated proteins are involved with T cell binding and activation.

Activation of the T cell entails the generation of a series of chemical signals (primarily cytokines) that result in the cell taking direct action or stimulating other cells of the immune system to act. In the case of class I MHC-antigen activation, CTL's proliferate and act to destroy infected cells presenting the same antigen. Killing an infected cell deprives a virus of life support and makes it accessible to antibodies, which finally eliminate it. In contrast, activation of T_h cells by class II MHC-antigen complexes does not destroy the antigen presenting cell (which is part of the host's defense system) but rather stimulates the T_h cell to proliferate and generate signals (again primarily cytokines) that affect various cells. Among other consequences, the signaling leads to B cell stimulation, macrophage activation, CTL differentiation and promotion of inflammation. This concerted response is relatively specific and is directed to foreign elements bearing the peptide presented by the class II MHC system.

When operating properly the immune response is surprisingly effective at eliminating microscopic pathogens and, to a lesser extent, neoplastic cells. In general, the complicated mechanisms for self-recognition are very efficient and allow a strong response to be directed exclusively at foreign antigens. Unfortunately, the immune system occasionally malfunctions and turns against the cells of the host thereby provoking an autoimmune response. Typically, autoimmunity is held to occur when the antigen receptors on immune cells recognize specific antigens on healthy cells and cause the cells bearing those particular substances to die. In many cases, autoimmune reactions are self-limited in that they disappear when the antigens that set them off are cleared away. However, in some instances the autoreactive lymphocytes survive longer than they should and continue to induce apoptosis or otherwise eliminete normal cells. Some evidence in animals and humans indicates that extended survival of autoreactive cells is implicated in at least two chronic autoimmune disorders, systemic lupus erythematosus and rheumatoid arthritis.

Other mechanisms of action are also thought to contribute to the development of various autoimmune disorders. For example, over the last few years it has become clear that the avidity of T cell-APC interactions dictates thymic learning and tolerance to self antigens. Accordingly, high avidity interactions lead to elimination of the T cell whereas low evidity interactions allow for maturation and exit from the thymus. Although this mechanism is effective in purging the immune system of autoreactivity, T cell precursors endowed with self reactivity could still be generated and migrate to the periphery if the autoantigen is sequestered and does not achieve effective levels of thymic presentation, is subjected to thymic crypticity, or is poorly presented. Moreover, superantigens capable of reacting with particular T cell receptors and events that could stimulate antigen mimicry, epitope spreading or peripheral loosening in peptide crypticity may trigger activation of those self-reactive T cells and cause antigen exposure. In any case, continuous supply of autoantigen and abundant generation of T cell receptor ligands (peptide-MHC complexes) are a likely mechanism of T cell aggressivity. Examples of such a spontaneous break in self-

tolerance include multiple sclerosis (MS), rheumatoid arthritis (possibly more than one mechanism) and type I diabetes all of which are thought to be T cell mediated autoimmune diseases.

Regardless of which mechanism is responsible for the corruption of the immune system, the results can be devastating to the individual. For example, multiple sclerosis is a chronic, inflammatory disorder that affects approximately 250,000 individuals in the United States. The inflammatory process occurs primarily within the white matter of the central nervous system and is mediated by T cells, B cells and macrophages which are responsible for the demyelination of the axons. Although the clinical course can be quite variable, the most common form is manifested by relapsing neurological deficits including paralysis, sensory deficits and visual problems.

Once immune cells have spread to the white matter of the central nervous system, the immune response is targeted to several different antigens on myelin. For example, there is a critical antibody response directed to myelin that activates the complement cascade with membrane attack complexes appearing in the spinal fluid. Further, T cells are targeted to certain key portions of various myelin antigens such as those presented on myelin basic protein (MBP) and proteolipid protein (PLP). The T cells in turn produce cytokines which then influence macrophages to attack the myelin and phagocytose large chunks of the myelin sheath. The concerted attack leads to areas of demyelination impairing salatory conduction along the axon and producing and the pathophysiologic defect. Multiple immune responses to several components of a supramolecular structure, like the myelin sheath in multiple sclerosis or the pyruvate dehydrogenase complex in primary biliary cirrhosis, are common in individuals with autoimmune diseases involving discrete organs.

Treatments for autoimmune diseases have met with varying levels of success. For example, it is often possible to correct organ-specific autoimmune disease through metabolic control. Where function is lost and cannot be restored, mechanical substitutes or tissue grafts may be appropriate. However, no effective treatments exist for several of the most disabling disorders including MS. While a number of compounds, including corticosterioids and modified beta interferon, can reduce some symptoms of MS, they have proven to have serious side effects or otherwise been shown to be less than desirable for long term use. Other avenues of treatment have shown promise but have yet to be shown effective.

In this respect, one promising treatment for MS is described in WO 96/16086, incorporated herein by reference, which discloses the use of peptide analogs of myelin basic protein (MBP). Compositions comprising these analogs are reportedly able to ameliorate symptoms of MS without excessive side effects. Moreover, use of peptide analogs to myelin constitutive proteins were also shown to be effective in treating the symptoms of experimental allergic encephalomyelitis (EAE), an organ specific immune disorder often used in mice as a model for MS. Specifically, reversal of EAE was achieved with a peptide analog derived from proteolipid (PLP) peptide (Kuchroo et al., *J. Immunol.* 153:3326-3336, 1994, incorporated herein by reference). It was shown that when the major TCR contacting residues within the naturally occurring PLP peptide were mutated, the resulting peptide analog bound MHC as well as the natural peptide yet does not activate PLP specific T cells. Instead the PLP analog inhibits *in vitro* activation of the T cells.

While peptide analogs represent an attractive approach to modulate the effector functions of aggressive T cells and ameliorate autoimmune diseases, several problems limit their effectiveness. For instance, only a few MHC-peptide complexes are available on the surface of a typical APC meaning a single complex may be required to serially trigger about 200 TCRs to activate the T cell. Where the autoantigen is continuously available for normal processing and presentation by the MHC system, it appears that very few surface MHC complexes would be available to bind the peptide analog. Further, as free peptides typically have very short half-lives, they are not readily incorporated and processed by the MHC-antigen presenting system, little will be naturally expressed on the APC. Due to the inefficient presentation, direct inhibition of the thousands of TCR's on each T cell likely require prohibitively high intracellular levels of free peptide. The turnover of cell surface MHC molecules also contributes to the short stay of complexes formed at the extracellular milieu (i.e. MHC class II molecules have been in the cell surface for some time before binding the extracellular peptide) while complexes formed in the endocytic compartment will reside for a normal period of time because they have just been translocated to the cell surface. Finally, as previously alluded to, administration of such synthetic epitopes or analogs is extremely problematic in view of the short half-life of peptides in the mammalian body. Between the short half-lives of the MHC complexes and the administered peptides, effective exposure is too brief to permit the induction of a satisfactory immune response further necessitating higher doses.

Accordingly, it is a general object of the present invention to provide methods and associated compositions for effectively modifying the immune system of a vertebrate to treat an immune disorder.

It is another object of the present invention to provide methods and compositions for the effective presentation of T cell receptor antagonists or agonists to modulate the cellular immune response in a subject in need thereof.

It is yet a further object of the present invention to provide methods and compositions for the treatment and amelioration of various immune disorders.

It is yet another object of the present invention to provide methods and compositions for the induction of T cell tolerance in neonates or infants.

It is still another object of the present invention to provide for the relief of pathological symptoms associated with autoimmune disorders including multiple sclerosis.

Summary of the Invention

These and other objectives are accomplished by the methods and associated compounds and compositions of the present invention which, in a broad aspect, provides for an Fc receptor mediated, endocytic delivery system. In selected embodiments the invention provides for the effective presentation of immunosuppressive factors which, in preferred embodiments may comprise T cell receptor antagonists or agonists. More particularly, the present invention provides methods, compounds and compositions to present immunosuppressive factors for the selective modification of an immune response in a vertebrate. In particularly preferred embodiments, the invention provides for Fc receptor mediated endocytic presentation of a selected T cell receptor antagonist or agonist to modulate an

immune response mounted against a specific antigen. As will be appreciated by those skilled in the art, the disclosed methods and compositions may be used to treat any physiological disorder related to the immune response of a vertebrate. For example, this ability to suppress selected components of the immune system may allow, among other things, for the treatment of autoimmune diseases, facilitation of tissue or organ transplants and the mitigation of symptoms produced by allergens. Moreover, the present invention further provides for the induction of tolerance in neonates and infants with regard to autoantigens.

In preferred aspects of the invention, the endocytic presentation of the selected immunosuppressive factor is facilitated through the use of an immunomodulating agent that is able to bind to the Fc receptor (FcR) of antigen presenting cells. Typically, the immunomodulating agent will comprise at least one immunosuppressive factor associated with at least one ligand capable of binding to a Fc receptor. Upon binding to the antigen presenting cell (APC) the immunomodulating agent will be internalized and processed by the APC's natural endocytic pathway. Preferably, the internalized immunosuppressive factor, which can be a T cell receptor antagonist or agonist, will then be associated with the newly synthesized endogenous MHC class II structures and presented at the surface of the APC. Those skilled in the art will appreciate that the immunosuppressive factors, while complexing with T cell receptors when bound to MHC class II structures, will not promote activation of the T cell. It will further be appreciated that hundreds of TCR's on each T cell must be triggered in order to activate the cell. Accordingly, efficient presentation of an appropriate TCR antagonist or agonist can prevent a previously primed T cell (i.e. one sensitized to a particular autoantigen) from activating and triggering an immune response despite competitive presentation of the naturally occurring autoantigen.

In a broad sense, the immunomodulating agents of the present invention may comprise any ligand (FcR ligand) that is capable of binding to, and being internalized by, the Fc receptor of an antigen presenting cell. That is, the FcR ligand may be any protein, protein fragment, peptide or molecule that effectively binds to a Fc receptor on the surface of any antigen presenting cell. Preferably, the FcR ligand will comprise or mimic at least some portion of a constant region of an immunoglobulin molecule and will not provoke an antigenic response in the subject. In selected aspects of the invention, the FcR ligand will comprise part or all of a constant region from an IgG molecule. Particularly preferred embodiments will employ FcR ligands comprising the entire constant region of a selected immunoglobulin molecule from the species to be treated. Of course, it will also be appreciated that binding to the Fc receptor may also be effected by ligands that comprise small fragments of a single constant region domains or non amino acid based molecular entities. In any case, the FcR ligand may be derived using modern pharmaceutical techniques such as directed evolution, combinatorial chemistry or rational drug design.

As previously alluded to, the compounds of the present invention further comprise an immunosuppressive factor associated with the FcR ligand to provide an immunomodulating agent. For the purposes of the instant invention the immunosuppressive factor can be any molecular entity that is capable of being processed by an APC and presented in association with class II MHC molecules on the cell surface. In particularly preferred embodiments the immunosuppressive factor comprises all or part of a T cell antagonist. For the purposes of this disclosure the term "antagonist" shall, in accordance with its normal meaning, comprise any substance that interferes with the

physiological action of another by combining with, and blocking, its receptor. More particularly, TCR antagonists are molecular entities that, in combination with class II MHC molecules, are capable of non-reactively associating with a T cell receptor and preventing that receptor from binding to its normal activating antigen ligand (i.e. an MHC-peptide agonist). Preferably, the TCR antagonist comprises a peptide or protein fragment that is an analog of the normal activating antigen agonist. In particularly preferred embodiments the TCR antagonist is an analog of a T cell epitope.

In other preferred embodiments the immunosuppressive factor may comprise a T cell agonist that forms a MHC complex which does not activate the primed TCR upon binding. For the purposes of the present disclosure, the term "agonist" shall be used in accordance with its commonly accepted biochemical meaning. In this regard it will be appreciated that, while the T cell agonist may be any molecule that provides the desired immunogenic result, the selected agonist will preferably comprise a peptide or protein fragment. Moreover, those skilled in the art will appreciate that immunomodulating agents comprising one or more T cell receptor agonists may be combined with immunomodulating agents comprising one or more T cell receptor antagonists to provide pharmaceutical formulations that may be used to selectively attenuate a patient's immune response.

In the disclosed compounds and associated methods, the FcR ligand is associated with the immunosuppressive factor to form an immunomodulating agent so that both are internalized by the APC at substantially the same time. This association may be in the form of two or more molecules bound to each other as with an antibody-antigen complex or, in preferred embodiments, may comprise the formation of a single chimeric molecule incorporating both the immunosuppressive factor (i.e. a TCR antagonist or agonist) and FcR ligand. For example, a selected TCR antagonist could be chemically linked to an FcR ligand region produced by proteolytic techniques (i.e. an Fc fragment). Other embodiments may comprise a normal immunoglobulin comprising an FcR ligand sterically bound to an antagonistic or agonistic peptide. Particularly preferred embodiments of the invention comprise chimeric immunoglobulins produced through genetic engineering techniques. In these compounds the FcR ligand (and usually the majority of the molecule) comprises one or more immunoglobulin constant regions while one or more of the variable regions is engineered to express a desired peptide TCR antagonist or TCR agonist. Those skilled in the art will appreciate that any combination of the aforementioned immunomodulating agents may be associated to form compositions of the present invention as can similar immunomodulating agents comprising different immunosuppressive factors. Moreover, as previously alluded to, mixtures or "cocktails" of various immunomodulating agents are specifically contemplated as falling within the scope of the present invention.

The disclosed compositions may be formulated using conventional pharmaceutical techniques and carriers and may be administered through the usual routes. However, the use of FcR mediated uptake of the immunomodulating agent avoids many of the problems associated with prior art compositions. More specifically, the methods of the present invention overcome many of the limitations associated with the administration of free peptide antagonists as disclosed in the prior art. Accordingly, efficient endocytic presentation of an immunosuppressive factor such as a TCR antagonist can generate significant levels of MHC-antagonist ligands to oppose abundant MHC-autoantigenic complexes that are generated in spontaneous immune disorders involving the continuous presentation

of an autoreactive antigen. As such, the invention may be used to treat any immune disorder that responds to the presentation of immunosuppressive factors. This is particularly true of T cell mediated autoimmune disorders including, for example, multiple sclerosis, lupis, rheumatoid arthritis, scleroderma, insulin-dependent diabetes and ulcerative colitis. In a like manner, the present invention can be used to selectively downregulate the immune system with respect to continuously presented agonists such as allergens. Further, the compounds and associated compositions of the present invention may be used to selectively suppress various components of the immune system to reduce the likelihood of tissue or organ rejection following transplant.

In addition, it has been surprisingly found that the compounds, compositions, and methods of the present invention may be used to induce tolerance to various autoantigens in neonates and infants. More particularly, the present invention further provides compositions and methods for conferring resistance in neonate or infant mammals to the induction of an autoimmune disease during adult life. In accordance with the teachings herein this neonatal tolerance is characterized by a lymph node deviation and unusual gamma interferon-mediated splenic energy upon challenge with the appropriate autoantigen. Further, in preferred embodiments the present invention may provide for the induction of the desired neonatal tolerance without the use of adjuvants (such as incomplete Freund's adjuvant).

Other objects, features and advantages of the present invention will be apparent to those skilled in the art from a consideration of the following detailed description of preferred exemplary embodiments thereof taken in conjunction with the figures which will first be described briefly.

Brief Description of the Drawings

Figs. 1A and 1B are schematic representations of chimeric immunoglobulin G (IgG) molecules illustrating the general features thereof and the inclusion of foreign peptides within the CDR 3 loop of the heavy chain variable region wherein Fig. 1A (Ig-PLP1) shows the insertion of a naturally occurring peptide PLP1 (agonist) derived from proteolipid protein while Fig. 1B (Ig-PLP-LR) illustrates an immunomodulating agent comprising the inclusion of a peptide analog (antagonist) to PLP1 termed PLP-LR;

Figs. 2A and 2B are graphical representations illustrating the capture of chimeric antibodies Ig-PLP1 and Ig-PLP-LR, which correspond to those shown in Figs. 1A and 1B respectively, using antibodies directed to the corresponding free peptides wherein Fig. 2A shows capture levels by antibodies directed to PLP1 and Fig. 2B shows capture levels by antibodies directed to PLP-LR with Ig-W, a wild type antibody, acting as a negative control;

Figs. 3A and 3B are graphs illustrating the presentation of Ig-PLP1 and Ig-PLP-LR (as well as positive and negative controls) to PLP1-specific T cell hybridomas 4E3 (Fig. 3A) and 5B6 (Fig. 3B) to determine the relative T cell activation potentials of the chimeric immunoglobulins as measured by IL-2 production;

Fig. 4 is a graphical representation illustrating the relative effectiveness of presenting PLP1 using the chimeric antibodies of the present invention (Ig-PLP1) versus the free peptide PLP1 or the native proteolipid protein (PLP) as measured by levels of IL-2 production following incubation with splenic SJL antigen presenting cells and PLP1 specific 4E3 T cell hybridoma;

Figs. 5A, 5B and 5C are graphical comparisons showing Ig-PLP-LR antagonism of PLP1 (5A), Ig-PLP1 (5B) and PLP (5C) mediated T cell activation as measured by IL-2 production by T cell hybridoma 4E3 in the presence of SJL splenic APCs that were previously incubated with the respective agonist and various levels of Ig-PLP-LR or controls;

Fig. 6 is a graph showing the relative antagonism of Ig-PLP2, Ig-PLP-LR and Ig-W as measured by the production of IL-2 by T cell hybridoma HT-2 in the presence of SJL splenic APCs that were previously incubated with native proteolipid protein in combination one of the aforementioned immunoglobulins;

Figs. 7A and 7B are graphs demonstrating the *in vivo* presentation of PLP1 following inoculation with Ig-PLP1 as measured by ^{3}H-thymidine incorporation by cells from the lymph node (7A) or the spleen (7B) wherein the illustrated values represent the ability of cells harvested from individual mice to generate a T cell response as measured by ^{3}H-thymidine incorporation when exposed to agonist PLP1 or the control peptide PLP2;

Figs. 8A and 8B are graphical representations showing the ability of Ig-PLP-LR to reduce the immune response to PLP1 peptide when co-administered with Ig-PLP1 as measured in murine cells from the lymph node (8A) or the spleen (8B) wherein the illustrated values represent the ability of cells harvested from individual mice to generate a T cell response as measured by ^{3}H-thymidine incorporation when exposed to PLP1;

Figs. 9A and 9B are graphs demonstrating that mice inoculated with a mixture of Ig-PLP-LR and Ig-PLP1 develop a more vigorous immune response to the peptide analog PLP-LR than peptide PLP1 as measured in cells from the lymph node (9A) or the spleen (9B) wherein the illustrated values represent the ability of cells harvested from individual subjects to generate a T cell response as reflected by ^{3}H-thymidine incorporation when exposed to either PLP1 peptide or the peptide analog PLP-LR.

Figs. 10A-10D are graphical representations of lymph node proliferative responses to immunization with Ig-PLP chimeras with mice individually tested in triplicate wells for each stimulator and where the indicated cpms represent the mean ± SD after deduction of background cpms;

Fig. 11 is a graphical representation of lymph node T cell proliferative response to co-immunization with Ig-PLP1 and Ig-PLPLR with stimulators comprising PPD, 5 μg/ml; PLP 1, PLP-LR, and PLP2 at 15 μg/ml;

Fig. 12 is a graphical representation of splenic proliferative T cells responses of mice immunized with Ig-W, Ig-PLP1, IG-PLP-LR and combinations thereof when stimulated with PLP1 (filled bars) and PLP-LR (hatched bars) in triplicate wells;

Figs. 13A-13C are graphical representations of IL-2 (13A), INFγ (13B), and IL-4 (13C) production by splenic cells of mice immunized with Ig-W, Ig-PLP1, Ig-PLP-LR and combinations thereof;

Figs. 14A-14D graphically illustrate proliferation of antigen experienced T cells from mice immunized with Ig-PLP1 (a and b) or Ig-PLP-LR (c and d) in CFA upon stimulation in vitro with PLP1 peptides, PLP-LR peptides and mixtures thereof;

Figs. 15A and 15B are graphical representations of IL-2 production by antigen experienced T cells immunized with Ig-PLP1 (15A) and Ig-PLP-LR (15B) upon in vitro stimulation with PLP1 peptide, PLP-LR peptide or mixtures thereof;

Figs. 16A and 16B graphically illustrate that neonatal mice injected with Ig-PLP1 and Ig-W resist induction of EAE with clinically derived curves shown for all mice (16A) and for surviving mice (16B);

Figs. 17A and 17B graphically show *in vivo* presentation of Ig-PLP1 by neonatal thymic (17A) and splenic (17B) antigen presenting cells following injection with Ig-PLP1 or Ig-W within 24 hours of birth;

Figs. 18A and 18B graphically illustrate lymph (18A) and splenic (18B) proliferative T cell response in mice injected with Ig-PLP1 or Ig-W shortly after birth upon stimulation with free PLP1, PLP2 or a negative control peptide corresponding the encephalitogenic sequence 178-191 of PLP;

Figs. 19A-19C graphically represent lymph node T cell deviation as measured by production of IL-2 (19A), IL-4 (19B), and INFγ (19C) in mice treated with Ig-PLP1 shortly after birth and stimulated with free PLP1 or PLP2;

Figs. 20A-20C graphically represent splenic T cell deviation as measured by production of IL-2 (20A), IL-4 (20B), and INFγ (20C) in mice treated with Ig-PLP1 shortly after birth and stimulated with free PLP1 or PLP2; and

Fig. 21 graphically illustrates cytokine mediated restoration of splenic T cell proliferation in mice injected with Ig-PLP1 shortly after birth, immunized with free PLP1 at seven weeks and stimulated with free PLP1 with the cells grown in control media (NIL) media with IL-12 and media with INFγ with the indicated cpms for each mouse representing the mean ± SD of triplicate wells.

Detailed Description of the Preferred Embodiment

While the present invention may be embodied in many different forms, disclosed herein are specific illustrative embodiments thereof that exemplify the principles of the invention. It should be emphasized that the present invention is not limited to the specific embodiments illustrated.

As previously alluded to, the present invention provides compounds, compositions and methods for selectively modifying the immune response of a vertebrate using an Fc receptor mediated endocytic delivery system. Essentially, any immunomodulating agent that can exploit this form of cellular uptake to downregulate the immune system is held to constitute part of the present invention. Among other forms, the immunomodulating agents of the invention may comprise single polypeptides, antigen-antibody complexes, chimeric antibodies or non-peptide based immunoactive compounds. In preferred embodiments the immunomodulating compounds disclosed herein will comprise at least one FcR ligand and at least one immunosuppressive factor that is capable of downregulating an immune response upon endocytic presentation. Particularly preferred embodiments of the invention comprise an immunomodulating agent wherein the immunosuppressive factor is a T cell receptor antagonist or agonist that is capable of binding with a receptor on the surface of a primed T cell but not capable of generating an immunogenic response. In such embodiments, the presented immunosuppressive factor will effectively compete with selected naturally occurring autoantigens thereby preventing the activation of the corresponding primed T cells and reducing the response generated. This selective suppression of the immune system may, among other indications, be used to treat

symptoms associated with immune disorders, including T cell mediated autoimmune disorders, allergies and tissue rejection in transplant operations.

Accordingly, in one embodiment the present invention comprises an immunomodulating agent for the endocytic presentation of an immunosuppressive factor on the surface of an antigen presenting cell of a vertebrate comprising at least one Fc receptor ligand and at least one immunosuppressive factor. Preferred embodiments comprise a Fc receptor ligand corresponding to at least a part of an immunoglobulin constant region domain while the immunosuppressive factor corresponds to at least one T cell receptor antagonist. Other preferred embodiments incorporate an immunosuppressive factor comprising a T cell receptor agonist. In particularly preferred embodiments the immunomodulating agent comprises a recombinant polypeptide or a chimeric antibody.

By exploiting FcR mediated uptake of the selected immunomodulating agent the present invention very cleverly uses the body's own metabolic pathways to downregulate harmful immune responses. More specifically, the present invention uses the fact that T cells only recognize and respond to foreign antigens only when attached to the surface of other cells. Selection of the appropriate immunomodulating agent or agents in accordance with the teachings herein provides for the efficient uptake of the administered compound. Following FcR mediated uptake, the natural endocytic pathway of antigen presenting cells provides for the effective presentation of the selected immunosuppressive factor complexed with the MHC class II molecules.

As described above, the two requisite properties that allow a cell to function as an antigen presenting cell for class II MHC-restricted helper T cell lymphocytes are the ability to process endocytosed antigens and the expression of class II MHC gene products. Most cells appear to be able to endocytose and process protein antigens. Accordingly, the determining factor appears to be the expression of class II MHC molecules. In this respect, the best defined antigen presenting cells for helper T lymphocytes comprise mononuclear phagocytes, B lymphocytes, dendritic cells, Langerhans cells of the skin and, in some mammals, endothelial cells. Of course it will be appreciated that different cells may be concentrated in different areas and may be involved in different stages of the T cell mediated immune response. In any case, the term "antigen presenting cell" or "APC" as used herein shall be held to mean any cell capable of inducing a T cell mediated immune response through the processing and surface presentation of an MHC class II-antigen complex. As such, the selected FcR ligand may interact with any of a number of different Fc receptors found on a variety of cell types to promote endocytosis of the immunomodulating agent. By way of example only, selected human Fc receptors that may be employed include the FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA or FcγRIIIB subfamilies.

More generally, in accordance with the present invention those skilled in the art will appreciate that any ligand capable of binding to an FcR complex and initiating endocytosis is compatible with the present invention and may be incorporated in the disclosed immunomodulating agents. Accordingly, FcR ligands may comprise, but are not limited to, peptides, proteins, protein derivatives or small molecular entities that may or may not incorporate amino acids. For example, small molecules derived using modern biochemical techniques such as combinatorial chemistry or rational drug design may be employed as long as they provide for the requisite APC uptake.

While it must be emphasized that any type of compatible molecule may be used, the FcR ligands of the present invention will preferably comprise one or more peptides. More preferably, the FcR ligand will comprise at least a part of a domain of a constant region of an immunoglobulin. In particularly preferred embodiments the FcR ligand will comprise one or more domains derived from a constant region of an immunoglobulin molecule. Those skilled in the art will appreciate that various immunoglobulin isotypes and allotypes may be employed as desired. For example, compatible FcR ligands may be selected from amino acid sequences corresponding to those found in the constant regions of IgG, IgE, IgA or IgM. Among other factors, selection of a particular isotype for use as a FcR ligand may be predicated on biochemical properties such as binding coefficients or low immunoreactivity in the species to be treated. Similarly, the selection of a single domain, fragment thereof or multiple domains may be determined based on biochemical factors or, ultimately, presentation efficiency.

Yet, efficient presentation via the endocytic pathway is typically not enough to selectively downregulate the immune response with regard to a particular antigen. Accordingly, immunomodulating agents of the present invention further comprise an immunosuppressive factor. In accordance with the scope of the present invention the immunosuppressive factor may be any compound that, when endocytically processed and presented on the surface of an APC in conjunction with a MHC class II complex, will downregulate the immune system. As such, immunosuppressive factors may comprise small molecules, peptides, protein fragments, or protein derivatives. In preferred embodiments the immunosuppressive factor acts as an antagonist when presented on the surface of the APC in that it interferes with the binding of a similarly presented agonist to a selected receptor. In particularly preferred embodiments the immunosuppressive factor comprises a T cell receptor antagonist that will associate with a T cell receptor without activating an immune response. Further, other embodiments of the invention comprise immunomodulating agents incorporating T cell receptor agonists that reduce the immune response to the subject autoantigen.

While any functionally compatible molecule may be used as an immunosuppressive factor in accordance with the present invention, those skilled in the art will appreciate that protein fragments or peptides are particularly suitable for use in the disclosed compounds and methods. Such molecules are readily processed by the normal endocytic pathways and are easily presented in concert with the MHC class II molecules on the surface of the antigen presenting cell. Moreover, as the majority of agonist compounds evoking an unwanted immune response are typically protein fragments, T cell receptors are usually most responsive to similar fragments whether they are agonists or antagonists. In particularly preferred embodiments, the immunosuppressive factor will be an analog of a selected peptide or protein fragment that is immunoreactive with a chosen T cell receptor.

"Peptide analogs" or "analogs," as used herein, contain at least one different amino acid in the respective corresponding sequences between the analog and the native protein fragment or peptide. Unless otherwise indicated a named amino acid refers to the L-form. An L-amino acid from the native peptide may be altered to any other one of the 20 L-amino acids commonly found in proteins, any one of the corresponding D-amino acids, rare amino acids, such as 4-hydroxyprofine, and hydroxylysine, or a non-protein amino acid, such as B-alanine and homoserine. Also included with the scope of the present invention are amino acids which have been altered by chemical means such

as methylation (e.g., a-methylvaline), amidation of the C-terminal amino acid by an alkylamine such as ethylamine, ethanolamine, and ethylene diamine, and acylation or methylation of an amino acid side chain function (e.g., acylation of the epsilon amino group of lysine).

Methods for selecting efficient peptide antagonists for treating multiple sclerosis (MS) are provided in PCT Publication No.: WO 96/16086 which has previously been incorporated into the instant application by reference. The disclosed methods may be used in concert with the present invention to provide effective immunosuppressive factors for incorporation in the disclosed immunomodulating agents. For example, using assays detailed below candidate peptide analogs may be screened for their ability to treat MS by an assay measuring competitive binding to MHC, T cell proliferation assays or an assay assessing induction of experimental encephalomyelitis (EAE). Those analogs that inhibit binding of the native autoreactive peptides, do not stimulate proliferation of native peptide reactive cell lines and inhibit the development of EAE (an experimental model for MS) by known autoantigens are useful for therapeutics. Those skilled in the art will appreciate that similar types of assays may be used to screen immunosuppressive factors for other native peptides (i.e. continuously presented autoantigens) and other immune disorders. In particularly preferred embodiments the selected immunosuppressive factors comprise analogs of T cell epitopes.

More generally, immunosuppressive factors may be derived for a number of diseases having a variety of immunoreactive agents without undue experimentation. For example, peptide analog antagonists or agonists may be generated for T cell epitopes on both proteolipid protein or myelin basic protein to treat multiple sclerosis. Similarly, T cell receptor antagonists or agonists may be derived from T cell epitopes of the pyruvate dehydrogenase complex to treat primary biliary cirrhosis. In both cases the derived immunosuppressive factors will be incorporated in a immunomodulating agent as described herein and administered to a patient in need thereof. Effective presentation of the immunosuppressive factor will selectively reduce stimulation of the autoreactive T cells by native peptide thereby relieving the symptoms of the subject immune disorder.

The selected immunosuppressive factor and FcR ligand, together comprising an immunomodulating agent, may be effectively administered in any one of a number of forms. More particularly, as described above, the immunomodulating agents of the present invention may combine any form of the respective elements that are functionally effective in selectively suppressing the immune response. For example, the immunomodulating agent may comprise a recombinant polypeptide or protein produced using modern molecular biology techniques. In such cases the FcR ligand may comprise a fragment of a single immunoglobulin region constant domain or, preferably, the entire constant region. In other embodiments the immunomodulating agent may comprise a sterically bound antibody-antigen complex wherein the antigen comprises a T cell receptor antagonist or agonist. Other preferred embodiments feature an immunomodulating agent comprising a chimeric antibody wherein an immunosuppressive factor is expressed on the Fab fragment. In still other embodiments the immunomodulating agent may comprise two covalently linked molecules which comprise a effective FcR ligand and immunosuppressive factor respectively.

Particularly preferred embodiments of the instant invention will employ recombinant nucleotide constructs to code for immunomodulating agents comprising a single fusion polypeptide. Those skilled in the art will appreciate

that standard genetic engineering technology can provide fusion proteins or chimeras that will comprise at least one FcR ligand and at least one immunosuppressive factor. As used herein the terms "chimera" or "chimeric" will be used in their broadest sense to encompass any polynucleotide or polypeptide comprising sequence fragments from more than one source. For example, a genetically engineered polypeptide incorporating a peptide TCR antagonist and a single Fc domain from an IgG molecule could properly be termed a chimeric or fusion protein. Similarly, a chimeric antibody may comprise a recombinant heavy chains engineered to incorporate a heterologous peptide immunosuppressive factor and a wild type light chains. For the purposes of the present invention, it is not necessary that the disparate regions be derived from different species. That is, a chimeric antibody may comprise human light and heavy chains and an engineered human TCR antagonist expressed in a CDR. Conversely, chimeric immunomodulating agents may comprise FcR ligands and immunosuppressive factors derived from different species such a human and mouse. As such, one aspect of the present invention comprises recombinant polynucleotide molecule encoding a polypeptide wherein said polynucleotide molecule comprises at least one nucleotide sequence corresponding to a Fc receptor ligand and at least one nucleotide sequence corresponding to an immunosuppressive factor. Preferably the immunosuppressive factor will correspond to a T cell receptor antagonist or agonist and the Fc receptor ligand corresponds to at least one constant region domain of an immunoglobulin. In a particularly preferred embodiment the polynucleotide molecule encodes a nucleotide sequence corresponding to an immunoglobulin heavy chain wherein a complementarity determining region has been at least partially deleted and replaced with a nucleotide sequence corresponding to a T cell receptor antagonist or agonist. Compositions comprising mixtures of immunosuppressive factors may also be used effectively in accordance with the teachings herein.

In any case, DNA constructs comprising the desired immunomodulating agents may be expressed in either prokaryotic or eukaryotic cells using techniques well known in the art. See, for example, Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1982 which is incorporated herein by reference. In preferred embodiments the engineered plasmid will be transfected into immortal cell lines which secrete the desired product. As known in the art, such engineered organisms can be modified to produce relatively high levels of the selected immunomodulating agent. Alternatively, the engineered molecules may be expressed in prokaryotic cells such as E. coli. Whatever production source is employed, products may be separated and subsequently formulated into deliverable compositions using common biochemical procedures such as fractionation, chromatography or other purification methodology and conventional formulation techniques.

Accordingly, another aspect of the invention comprises a method for producing an immunomodulating agent for the endocytic presentation of an immunosuppressive factor on the surface of an antigen presenting cell of a vertebrate comprising the steps of:

a. transforming or transfecting suitable host cells with a recombinant polynucleotide molecule comprising a nucleotide sequence which encodes a polypeptide comprising at least one Fc receptor ligand and at least one immunosuppressive factor;

b. culturing the transformed or transfected host cells under conditions in which said cells express the recombinant polynucleotide molecule to produce said polypeptide wherein the polypeptide comprises at least a part of an immunomodulating agent; and

c. recovering said immunomodulating agent.

Similarly, another aspect of the invention comprises transfected or transformed cells comprising a recombinant polynucleotide molecule encoding a polypeptide wherein the polypeptide comprises at least one Fc receptor ligand and at least one immunosuppressive factor.

In both of the preceding aspects, the immunosuppressive factor is preferably a T cell receptor antagonist or agonist and the Fc receptor ligand preferably comprises at least part of an immunoglobulin constant region domain. More preferably, the immunomodulating agent comprises a poly peptide or chimeric antibody wherein at least one complementarity determining region (CDR) has been replaced with a T cell receptor antagonist or agonist.

It will further be appreciated that the chimeric antibodies, polypeptides and other constructs of the present invention may be administered either alone, or as pharmaceutical composition. Briefly, pharmaceutical compositions of the present invention may comprise one or more of the immunomodulating agents described herein, in combination with one or more pharmaceutically of physiologically acceptable carriers, diluents or excipients. Such composition may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like, carbohydretes such as glucose, mannose, sucrose or dextrans, mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (e.g. aluminum hydroxide) and preservatives. In addition, pharmaceutical compositions of the present invention may also contain one or more additional active ingredients, such as, for example, cytokines like B-interferon.

In this respect a further aspect of the present invention comprise pharmaceutical compositions for the endocytic presentation of an immunosuppressive factor on the surface of an antigen presenting cell of a vertebrate comprising at least one immunomodulating agent and a pharmaceutically ecceptable carrier, said at least one immunomodulating agent comprising at least one Fc receptor ligand and at least one immunosuppressive factor. Similarly, the invention comprises methods for the preparation of a pharmaceutical composition to treat an immune disorder comprising combining at least one immunomodulating agent with a physiologically acceptable carrier or diluent wherein said immunomodulating agent comprises at least one Fc receptor ligand and at least one immunosuppressive factor. In both of these aspects the immunosuppressive factor may comprise a T cell receptor antagonist or agonist and the Fc receptor ligand may comprise at least part of a immunoglobulin constant region domain. Preferably, the immunomodulating agent will be in the form of a recombinant polypeptide or a chimeric antibody.

As indicated above, immunomodulating agents comprising chimeric antibodies are a particularly preferred aspect of the invention. Such antibodies may be formed by substituting a immunosuppressive factor, typically a peptide TCR antagonist, for at least part of one or more of the complementarity determining regions (CDR). As will be described more fully in the Examples below, the nucleotide sequence coding for the heavy chain may be engineered to replace all or part of at least one CDR with a peptide analog of all or part of an autoantigen. Upon expression by the proper cell line, the recombinant heavy chains can complex with wild type light chains to form an

immunoreactive tetramer displaying two immunosuppressive factors. Those skilled in the art will appreciate that the immunoglobulin molecules may be selected from the species to be treated so as to minimize the generation of a harmful immune response (i.e. a human anti-mouse response). As the constant region of the selected immunoglobulin is essentially unmodified, this form of immunomodulating agent is readily endocytosed allowing for effective presentation of the associated immunosuppressive factor.

In other forms, the immunomodulating agents of the present invention may comprise an antigen-antibody complex wherein the antigen is an immunosuppressive factor. It will be appreciated that modern immunological techniques may be used to generate and purify the desired antibodies which are preferably monoclonal. By way of example only, a selected peptide antagonist or agonist (i.e. an analog of a peptide autoantigen) may be injected into a mouse to provide immunoreactive cells which may then be harvested and immortalized using standerd methods. If desired, the murine monoclonal may be "humanized" using conventional recombinant procedures leaving e small murine variable region expressed on an otherwise human immunoglobulin that will not provoke a harmful immune response in a patient. In any case, the monoclonal antibody is complexed with the immunosuppressive factor to form the desired immunomodulating agent which may then be formulated and administered as described ebove. With the intact constant region forming the FcR ligand, phagocytation should be relatively rapid and presentetion of the attached immunosuppressive factor efficient.

Although embodiments may comprise the Fc receptor ligands corresponding to the entire constant region, it must be emphasized that the present invention does not require that the administered immunomodulating egent comprise an intact immunoglobulin constant region. Rather, any FcR ligand that can bind to the FcR and undergo endocytosis may be used in conjunction with the selected immunosuppressive factor. Specifically, single domains of constant regions or fragments thereof may be combined with peptide antagonists to form monomeric polypeptides (heving a single amino acid chain) that can suppress the immune system in accordance with the teachings herein. Such fusion proteins may be constructed which, having the minimum effective FcR ligand and/or immunosuppressive factor, may be much more stable thereby facilitating delivery and possibly increasing bioavailability. Moreover, these engineered proteins may be able to be administered over a period of time without provoking an immune response as is seen when administering whole antibodies of heterologous species. As such, relatively small chimeric polypeptides may prove to be effective immunomodulating agents.

Similarly, non-peptide based molecular entities may prove to be efficient FcR ligands, immunosuppressive factors or, in combination, immunomodulating agents. Those skilled in the ert will eppreciate thet molecuier entities (peptide based or non-peptide based) that function effectively in e selected role (i.e. FcR ligand) may be provided using current procedures such as combinatorial chemistry, directed evolution or rational drug design. For example, it may be possible to use rational drug design to fashion a small non-peptide molecular entity that effectively binds to a previously elucidated Fc receptor. The derived FcR ligand may then be covalently linked (or otherwise reversibly associated) with an immunosuppressive factor such as a peptide antagonist to provide an immunomodulating agent that exhibits particular stability or other desirable traits.

Whatever form of immunomodulating agent selected the compositions of the present invention may be formulated to provide desired stability and facilitate the selected form of administretion. For example, the compositions may be administered using all the conventional routes including, but not limited to, oral, vaginal, aural, nasal, pulmonary, intravenous, intracranial, intraperitoneal, subcutaneous, or intramuscular administration. Within other embodiments of the invention, the compositions described herein may be administered as part of a sustained release implant. Within yet other embodiments, compositions of the present invention may be formulated as a lyophilizate or spray dried formulation, utilizing appropriate excipients which provide stability as a lyophilizate, and subsequent to rehydration.

The present invention is useful for the treatment of any vertebrate comprising an immune system subject to down regulation. The invention is particularly useful in those vertebrates such as mammals that possess cellular immune responses. In preferred embodiments the vertebrate to be treated will be in a neonatel or infant state.

In this respect, a further aspect of the invention comprises a method for treating en immune disorder comprising administering to a patient a therapeutically effective amount of a pharmaceutical composition comprising an immunomodulating agent in combination with a physiologically acceptable carrier or diluent wherein said immunomodulating agent comprises at least one Fc receptor ligand and at least one immunosuppressive factor. For this aspect, the immunosuppressive factor may comprise a T cell receptor antegonist and the Fc receptor ligand may comprise at least part of a immunoglobulin constant region domain. As previously alluded to, the immunomodulating agent will preferably be in the form of a recombinant polypeptide or a chimeric antibody. The methods may be used treat immune disorders comprising autoimmune disorders, allergic responses end transplant rejection and are particulerly useful in treating autoimmune disorders selected from the group consisting of multiple sclerosis, lupis, rheumatoid arthritis, scleroderma, insulin-dependent diabetes and ulcerative colitis.

As discussed above, the compositions, compounds and methods of the present invention are particularly useful for inducing tolerance in neonatal or infant mammals thereby preventing or reducing future autoimmunity. The term "infant" as used herein, refers to a human or non-human mammal during the period of life following birth wherein the immune system has not yet fully matured. In humans, this period extends from birth to the age of about nine months while in mice, this period extends from birth to about four weeks of age. The terms "newborn" and "neonate" refer to a subset of infant mammals which have essentially just been born. Other charecteristics associated with "infants" according to the present invention include an immune response which has (i) susceptibility to high zone tolerance (deletion/anergy of T cell precursors, increased tendency for apoptosis); (ii) a Th_2 biased helper response (phenotypical particularities of neonatal T cells; decreased CD40L expression on neonatal T cells); (iii) reduced magnitude of the cellular response (reduced number of functional T cells; reduced antigen-presenting cell function); and (iv) reduced magnitude and restricted type of humoral response (predominance of IgM^{low}, IgD^{low}, B cells, reduced cooperation between Th and B cells). In specific nonlimiting embodiments of the invention the disclosed immunmodulating agents may be administered to an infant mammal wherein maternal antibodies remain present in detectable amounts. In a related embodiment, the pregnant mother may be inoculated with the disclosed compositions so as to produce the desired T cell tolerance in the fetus. In any case the induced T cell tolerance

may confer resistance to the later development of an autoimmune disease associated with the administered immunomodulating agent.

Regardless as to whether the subject is an infant or full grown, the pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patients disease. Within particularly preferred embodiments of the invention, the pharmaceutical compositions described herein may be administered at a dosage ranging from 1μg to 50 mg/kg, although appropriate dosages may be determined by clinical trials. Those skilled in the art will appreciate that patients may be monitored for therapeutic effectiveness by MRI or signs of clinical exacerbation.

Following administration, it is believed that the immunomodulating agent binds to one or more Fc receptors present on the surface of at least one type of antigen presenting cell. Those skilled in the art will appreciate that selection of the FcR ligand will, at least to some extent, determine which class of Fc receptor is used to internalize the immunomodulating agent. That is, a FcR ligand corresponding to an IgG constant region will be bound by a different class of Fc receptor than a FcR ligand corresponding to an IgE constant region. Moreover, as different classes of Fc receptors are expressed on different types of antigen presenting cells it is possible to present the immunosuppressive factor on selected APCs. For example, an FcR ligand corresponding to an IgG constant region is likely to be endocytosed by a macrophage or neutrophil and presented accordingly. This is of interest in that certain APCs are more efficient at presenting various types of antigens which, in turn, may influence which T cells are activated.

In any case, the entire immunomodulating agent is subjected to receptor mediated endocytosis by the APC and usually becomes localized in clathrin-coated vesicles. After internalization, the immunomodulating agent is processed for eventual presentation at the surface of the APC. Processing generally entails vesicle transport of the immunomodulating agent to the lysosome, an organelle comprising an acidic pH and selected enzymes including proteases. Here the immunomodulating agent is digested to provide a free immunosuppressive factor which, for the purposes of the instant invention, may be in the form of a peptide. In such cases average peptide lengths may be, for example, on the order of 5 to 30 amino acids. Following digestion, at least some of the immunomodulating agent fragments, including the immunosuppressive factor fragment, are associated with MHC class II molecules in exocytic vesicles. The MHC class II-immunosuppressive factor complex is then transported to the surface of the APC and presented to helper T cells.

As pointed out above, preferred embodiments of the invention use a TCR antagonist as the immunosuppressive factor presented in concert with the class II MHC molecules. Accordingly, such antagonists (which may be peptide analogs) will be used for the purposes of the following discussion. However, it must be emphasized that the present invention may be used for the receptor mediated endocytic presentation of any immunosuppressive factor that downregulates an immune response. As such, T cell receptor agonists which provide the desired reduction in immunogenic response may be used as immunosuppressive factors and are in the purview of the present invention.

Accordingly, by way of example only, a T cell may have previously been sensitized to an autologous peptide agonist corresponding to a fragment of myelin basic protein. In multiple sclerosis this autoagonist is continuously presented thereby activating an immune response directed to constituents of the myelin sheath. More particularly, the sensitized individual T cells express thousands of receptors which selectively bind to the presented autoagonist and signal the cell. When enough of the receptors are bound, the sensitized T cell acts to mount a response i.e. secrete interleukin. In the cases where a TCR antagonist is presented in concert with MHC class II molecules the T cell will recognize the presented complex but will not be activated.

Thus, in accordance with the present invention, efficient endocytic presentation of an immunosuppressive factor (i.e. an antagonist) inhibits agonist-TCR binding through competition for the receptors. That is, the presented TCR antagonist binds effectively to the TCR of a sensitized T cell thereby precluding binding of a presented autoantigen or fragment thereof. Yet, unlike an autoantigen-TCR complex, the immunosuppressive factor-TCR complex does not signal the T cell to mount a response. Thus, the binding of the immunosuppressive factor (non-reactive agonist or antagonist) can prevent a T cell from binding enough autoantigen to reach the threshold activation level that induces the cell to act. Hence, a harmful immune response to the continuously presented autoantigen comprising a natural agonist is averted.

Presentation of the following non-limiting Examples will serve to further illustrate the principles of the present invention. In this regard, a list of abbreviations and corresponding definition used throughout the following discussion and the Examples is provided:

MBP: myelin basic protein, has been implicated in the etiology of multiple sclerosis;

PLP: proteolipid protein, has been implicated in the etiology of multiple sclerosis;

PLP1: a peptide fragment of PLP comprising aa residues 139-151;

PLP-LR: a peptide analog of PLP1, does not activate PLP1 pulsed cells;

PLP2: a peptide fragment of PLP comprising aa residues 178-191;

Ig-W: an Ig construct (used herein as a control) comprising the heavy chain variable region of the anti-arsonate antibody 91A3, linked to a Balb/cγ2b constant region, and the parental 91A3 kappa light chain;

Ig-PLP1: the same construct as Ig-W except that the heavy chain CDR3 was replaced with aa residues 139-151 of PLP;

Ig-PLP-LR: the same construct as Ig-W except that the heavy chain CDR3 was replaced with a peptide analog of aa residues 139-151 of PLP;

Ig-HA: (used as a control herein) the same construct as Ig-W except that the heavy chain CDR3 was replaced with aa residues 110-120 of influenza virus HA;

PPD: purified protein derivative, whole *Mycobacterium tuberculosis* extract used as a control activator.

For obvious practical and moral reasons, initial work in humans to determine the efficacy of experimental compositions or methods with regard to many diseases is infeasible. Thus, during early development of any drug it is standard procedure to employ appropriate animal models for reasons of safety and expense. The success of implementing laboratory animal models is predicated on the understanding that immunodominant epitopes are

frequently active in different host species. Thus, an immunogenic determinant in one species, for example a rodent or pig, will generally be immunoreactive in a different species such as in humans. Only after the appropriate animal models are sufficiently developed will clinical trials in humans be carried out to further demonstrate the safety and efficacy of a vaccine in man. Accordingly, for purposes of explanation only and not for purposes of limitation, the present invention will be primarily demonstrated in the exemplary context of mice as the mammalian host. Those skilled in the art will appreciate that the present invention may be practiced with other mammalian hosts including humans and domesticated animals.

In this respect, experimental encephalomyelitis (EAE), which is used as an animal model for MS, can be induced in susceptible strains of mice with myelin autoantigens such as PLP and myelin basic protein (MBP). The encephalitogenic activity of these proteins correlates with the presence of peptides which induce in vivo class II restricted encephalitogenic T cells and consequently EAE. The peptide corresponding to aa residues 139-151 of PLP (PLP1) is encephalitogenic in H-2s SJL mice, and T cell lines specific for PLP1 transfer EAE into naive animals. Although the target antigen(s) in human MS is still debatable, the frequency of T cells specific for myelin proteins are higher in MS patients than in normal subjects. Silencing those myelin-reactive T cells may be a logical approach to reverse MS. As such, this model will be used to demonstrate the advantages of the present invention.

Example I

Preparation of Peptides

For the purposes of this application the amino acids are referred to by their standard three-letter or one-letter code. Unless otherwise specified, the L-form of the amino acid is intended. When the 1-letter code is used, a capital letter denotes the L-form and a small letter denotes the D-form. The one letter code is as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.

All peptides used in the following examples were produced by Research Genetic, Inc. (Huntsville, Alabama) using solid state methodology and purified on HPLC columns to >90% purity using conventional methods. PLP1 peptide (HSLGKWLGHPNKF: SEQ. ID No. 1) encompasses an encephalitogenic sequence corresponding to aa residues 139-151 of naturally occurring proteolipid protein. PLP-LR (HSLGKLLGRPNKF:SEQ. ID No. 2) is an analog of PLP1 in which Trp144 and His147 were replaced with Leu end Arg (underlined), respectively. PLP1 and PLP-LR bind well to I-A^s class II molecules (i.e. an MHC class II structure produced by a specific strain of mice). PLP2 peptide (NTWTTCQSIAFPSK:SEQ. ID No. 3) encompasses an encephalitogenic sequence corresponding to aa residues 178-191 of PLP. This peptide also binds to I-A^s class II molecules and induces EAE in SJL mice. HA peptide (sequence not shown) corresponds to aa residues 110-120 of the hemagglutinin of the Influenza virus. HA binds to I-E^d class II molecules and is used here as control peptide.

Example II

Production of Murine Chimeric Immunoglobulins Comprising Exogenous Peptides

Two immunoglobulin-peptide chimeras, designated Ig-PLP1 and Ig-PLP-LR and shown schematically in Figure 1, were constructed to express peptides PLP1 and PLP-LR as described in Example 1. In both cases, the heavy chain CDR 3 loop was deleted and replaced with nucleotide sequences coding for the selected peptide. Conventional DNA sequencing analysis indicated insertion of peptide nucleotide sequences in the correct reading frame.

The genes used to construct these chimeras include the gene coding for the BALBK IgG_2b constant region as described by Gillian et al., *Cell*. 33:717,1983, the gene coding for the 91A3 heavy chain variable region as described by Ruthban et al., *J. Mol. Bio.*, 202:383-398, 1988, and the gene coding for the entire 91A3 kappa light chain as described by Gary et al., *Proc. Natl. Acad. Sci.*, 84:1085-1089, 1987, all of which are incorporated herein by reference. The procedures for deletion of the heavy chain CDR3 region and replacement with nucleotide sequences coding for PLP1 and PLP-LR are similar to those described by Zaghouani et al. *J.Immunol.* 148: 3604-3609, 1992 and incorporated herein by reference, for the generation of Ig-NP a chimera carrying a CTL epitope corresponding to aa residues 147-161 of the nucleoprotein of PR8 influenza A virus. The same reference reports that the CDR3 of the 91A3 IgG is compatible for peptide expression, and that both class I and class II-restricted epitopes have been efficiently processed and presented to T cells when grafted in place of the naturally occurring segment.

Briefly, The $91A3V_H$ gene was subcloned into the EcoRI site of pUC19 plasmid and used as template DNA in PCR mutagenesis reactions to generate $91A3V_H$ fragments carrying PLP1 ($91A3V_H$-PLP1) and PLP-LR ($91A3V_H$-PLP-LR) sequences in place of CDR3. Nucleotide sequencing analysis indicated that full PLP1 and PLP-LR sequences were inserted in the correct reading frame (not shown). The $91A3V_H$-PLP1 and $91A3V_H$-PLP-LR fragments were then subcloned into the EcoRI site of pSV2-gpt-Cγ2b in front of the exons coding for the constant region of a Balb/cγ2b which generated pSV2-gpt-$91A3V_H$-PLP1-Cγ2b and pSV2-gpt-$91A3V_H$-PLP1-LR-Cγ2b plasmids, respectively. These plasmids were then separately cotransfected into the non-Ig producing SP2/0 B myeloma cells with an expression vector carrying the parental 91A3 light chain, pSV2-neo-91A3L. Transfectants producing Ig chimeras were selected in the presence of geneticin and mycophenolic acid. Transfectants were cloned by limiting dilution and final clones secreted 1 to 4 μg/mL of Ig-PLP1 or Ig-PLP-LR (collectively, the Ig-PLP chimeras). The selected cell lines, designated Ig-PLP1-9B11 and Ig-PLP-LR-21A10, are maintained in permanent storage in the inventor's laboratory.

Chimeric and wild-type antibodies were also used as controls. For example Ig-HA, an IgG molecule carrying in place of the D segment the HA110-120 T helper epitope from the HA of influenza virus that differs from Ig-PLP1 and Ig-PLP-LR only by the peptide inserted within CDR3. Ig-W is the product of unmodified (wild-type) $91A3V_H$ gene, Balb/cγ2b constant region and 91A3 kappa light chain. Therefore it differs from Ig-PLP1 and Ig-PLP-LR in the CDR3 region which comprises the parental D segment. Finally, Ig-PLP2, is a chimeric antibody that carries within the heavy chain CDR3 loop aa residues 178-191 of PLP. Conventional cloning, sequencing, and purification procedures were used to generate the appropriate cell lines and are similar to those described by Zaghouani et al. (previously cited)

and those previously used to generate Ig-HA, Zaghouani et al., *Science*. 259:224-227, 1993 also incorporated herein by reference.

Large scale cultures of transfectants were carried out in DMEM media containing 10% iron enriched calf serum (Intergen, New York). Ig-PLP chimeras were purified from culture supernatant on columns made of rat-anti-mouse kappa chain mAb and coupled to CNBr activated Sepharose 4B (Pharmacia). Rat-anti-mouse kappa chain mAb (RAM 187.1 or ATCC denotation, HB-58) and mouse anti-rat kappa light chain mAb (MAR 18.5 or ATCC denotation, TIB 216) were obtained from the ATCC. These hybridomas were grown to large scale and purified from culture supernatant on each other. The rat anti-mouse kappa mAb was used to prepare the columns on which the Ig-PLP chimeras were purified from culture supernatant. To avoid cross contamination separate columns were used to purify the individual chimeras.

Example III

Purification of Proteolipid Protein

Native proteolipid protein or PLP was purified from rat brain according to the previously described procedure of Lees et al., in Preparation of Proteolipids, *Research Methods in Neurochemistry*, N. Marks and R. Rodnight, editors. Plunemum Press, New York, 1978 which is incorporated herein by reference.

Briefly, brain tissue was homogenized in 2/1 v/v chloroform/methanol, and the soluble crude lipid extract was separated by filtration through a scintered glass funnel. PLP was then precipitated with acetone and the pellet was redissolved in a mixture of chloroform/methanol/acetic acid and passed through an LH-20-100 sephadex column (Sigma) to remove residual lipids. Removal of chloroform from the elutes and conversion of PLP into its apoprotein form were carried out simultaneously through gradual addition of water under a gentle stream of nitrogen. Subsequently, extensive dialysis against water was performed to remove residual acetic acid and methanol.

Example IV

Production of Rabbit Anti-Peptide Antibodies

PLP1 and PLP-LR peptides prepared in Example I were coupled to KLH and BSA as described in Zaghouani et al., *Proc. Natl. Acad. Sci USA*. 88:5645-5649, 1991 and incorporated herein by reference. New Zealand white rabbits were purchased from Myrtle's Rabbitry (Thompson Station, TN). The rabbits were immunized with 1 mg peptide-KLH conjugates in complete Freund's adjuvant (CFA) and challenged monthly with 1 mg conjugate in incomplete Freund's adjuvant (IFA) until a high antibody titer was reached. The peptide-BSA conjugates were coupled to sepharose and used to purify anti-peptide antibodies from the rabbit anti-serum.

Example V

Characterization of Rabbit Anti-Peptide Antibodies

Capture radioimmnoassays (RIA) were used to assess expression of PLP1 and PLP-LR peptides on an IgG molecule using Ig-PLP1 and Ig-PLP-LR made as described in Example II.

Microtiter 96-well plates were coated with the rabbit anti-peptide antibodies made in Example IV (5 μg/mL) overnight at 4°C and blocked with 2% BSA in PBS for 1 hour at room temperature. The plates were then washed 3 times with PBS, and graded amounts of Ig-PLP1 and Ig-PLP-LR were added and incubated for 2 hours at room temperature. After 3 washes with PBS, the captured Ig-PLP1 and Ig-PLP-LR were detected by incubating the plates with 100 x 10^3 cpm ^{125}I-labeled rat anti-mouse kappa mAb for 2 hours at 37°C. The plates were then washed 5 times with PBS and counted using an LKB gamma counter. Shown are the mean ± SD of triplicates obtained with 27 μg/mL of chimeras.

As shown in Figure 2, the rabbit antibodies directed to synthetic PLP1 and PLP-LR peptides recognized the chimeric antibodies Ig-PLP1 and Ig-PLP-LR produced in Example II. More specifically, when Ig-PLP1 and Ig-PLP-LR were incubated on plates coated with rabbit anti-PLP1 they were captured in significant quantity and bound labeled rat anti-mouse kappa chain mAb (Fig. 2A). Similarly, both Ig-PLP1 and Ig-PLP-LR were captured by rabbit anti-PLP-LR (Fig. 2B). Conversely, Ig-W, the wild type 91A3 murine antibody without an exogenous peptide and an IgM control antibodies (not shown), did not show significant binding to the rabbit antibodies. Ig-PLP1 bound to both anti-PLP1 and anti-PLP-LR better than did Ig-PLP-LR, indicating that structural differences affected accessibility of the peptides to the rabbit antibodies. Further, the results shown in Figure 2 indicate that peptide expression on the chimeras did not alter heavy and light chain pairing because the rabbit antibodies bind to the PLP peptide on the heavy chain and the labeled rat anti-mouse kappa binds on the light chain.

Example VI

Antigen Specific T Cell Line Proliferation Assays

PLP1-specific T cell hybridomas 5B6 and 4E3 and the IL-2 dependent HT-2 T helper cells were obtained from The Eunice Kennedy Shriver Center, Waltham, MA. The 5B6 and 4E3 T cells recognize the peptide PLP1 in association with I-A^S class II MHC and produces IL-2 when incubated with it as reported by Kuchroo et al., *J. Immunol.* 153:3326-3336, 1994 which is incorporated herein by reference. Conversely, Kuchroo et al. report that when stimulated with PLP1 and then with PLP-LR both 5B6 and 4E3 cells no longer produce IL-2. Similarly, stimulation of T cell hybridomas with PLP1 in the presence of PLP-LR apparently inhibits IL-2 production.

Using substantially the same technique as Kuchroo et al., activation of the T cell hybridomas for various agonists was performed as follows. Irradiated (3,000 rads) splenocytes from SJL mice were used as antigen presenting cells (APCs) for this Example. The irradiated splenocytes were incubated in 96-well round bottom plates (5 x 10^5 cells/well/50 μl) with graded concentrations of antigens (100 μl/well). After one hour, T cell hybridomas, i.e. 5B6 or 4E3 (5 X 10^4 cells/well/50 μl) were added and the culture was continued overnight. Activation (or proliferation) of the T cells was assessed by measuring production of IL-2 in the culture supernatant. This was done

by ^{3}H-thymidine incorporation using the IL-2 dependent HT-2 cells. That is, when IL-2 is present (i.e. secreted by activated T cells) the HT-2 cells proliferate, incorporating labeled thymidine from the surrounding media.

The culture media used to carry out these assays was DMEM supplemented with 10% FBS, 0.05 mM 2-mercaptoethenol, 2 mM glutamine, 1 mM sodium puryvate and 50 μg/mL gentamycin sulfate. Briefly, culture supernatants (100 μl/well) were incubated with HT-2 cells (1x 10^4 cells/well/100 μl) in 96-well flat bottom plates for 24 hours. Subsequently 1 μCi ^{3}H-thymidine was added per well and the culture was continued for an additional 12-14 hours. The cells were then harvested on glass fiber filters and the non incorporated ^{3}H-thymidine was washed away. Incorporated thymidine was then counted using the trace 96 program and an Inotech β counter. It will be appreciated that those wells containing higher levels of IL-2 (secreted by the activated T cell hybridoma lines) will induce higher levels of HT-2 cell proliferation and register increased levels of ^{3}H-thymidine incorporation.

The results of the aforementioned assay using two different T cell lines are shown in Figure 3. Specifically, T cell hybridomas 4E3 (Fig. 3A) and 5B6 (Fig. 3B) produced substantial levels of IL-2 following stimulation by APCs previously incubated with Ig-PLP1, PLP1 and native PLP. The negative controls Ig-W, Ig-HA, and PLP2 peptide did not induce the production of IL-2 by the T cells. Similarly, both Ig-PLP-LR and PLP-LR peptide did not stimulate 5B6 and 4E3 to produce significant levels of IL-2. These last results are not unexpected because the PLP-LR peptide is known to negate rather than stimulate IL-2 production. The concentration of antigen was 0.1 μM for Ig-PLP1, Ig-PLP-LR, Ig-HA, and Ig-W; 1 μM for PLP1, and PLP2 peptides; and 1.7 μM for PLP. Each value represents the mean ± SD of triplicate wells.

These results indicate that Ig-PLP1 was presented to the T cell hybridomas in a manner conducive to activation. Steric hindrance appears to preclude the simultaneous direct binding of the whole antibody to the MHC structure and TCR. As T cells will not react to soluble proteins, it appears that the PLP1 peptide was released from the Ig by endocytic processing and bound MHC class II I-A^s molecules. Accordingly, the regions flanking the PLP1 peptide do not appear to interfere with the endocytic processing of Ig-PLP1 or the binding of the PLP1 peptide to the MHC class II structure.

Example VII

Presentation of PLP1 Peptide to T Cells Via Ig-PLP1

In spontaneous immune disorders, exposure and continuous endocytic presentation of an autoantigen may generate significant levels of MHC-autoantigen complexes. Currently many immune diseases lack an effective *in vitro* model for replicating this continuous presentation affording a serious impediment to the development of effective treatments. Due to relatively inefficient internalization mechanisms or the previously discussed limitations relating to free peptides, relatively high levels of natural antigens are required to provide the desired stimulation. Accordingly, one aspect of the present invention is to provide an *in vitro* model for the continuous endocytic presentation of agonist ligands.

More particularly, the present invention provides methods for the effective *in vitro* endocytic presentation of a T cell antagonist comprising the steps of:

a. providing a medium comprising a plurality of antigen presenting cells expressing Fc receptors; and

b. combining said medium with a immunomodulating agent containing composition wherein the composition comprises an immunomodulating agent having at least one Fc receptor ligand and at least one immunosuppressive factor and a compatible carrier.

Preferably the immunosuppressive factor will be at least one T cell receptor antagonist and the Fc receptor ligand will be at least part of a immunoglobulin constant region domain. Further, in preferred aspects of the invention the immunomodulating agent will comprise a recombinant polypeptide or a chimeric antibody.

In this respect, Ig-PLP1 (or eny immunoglobulin associated agonist) may be used for the purpose of establishing a peptide delivery system that could efficiently operate through the endocytic pathway and generate high levels of agonist ligands such that it provides an *in vitro* system to investigate the immune system. In particular, the disclosed system may be used to investigate antagonism in a situation similar to the *in vivo* presentation of autoantigens.

To demonstrate that immunoglobulin associated agonists may be used to mimic continuous endocytic presentation of antigens, T cell activation assays were performed with free PLP1 peptide, native PLP, and Ig-PLP1. The results of the assays are shown in Fig. 4.

Specifically, different concentrations of the three antigens (i.e. agonists) were incubated with irradiated SJL/J splenocytes which were subsequently associated with 4E3 T cell hybridomas. IL-2 production was measured by ^{3}H-thymidine incorporation using the IL-2 dependent HT-2 cells as described in Example VI. Each point represents the mean of triplicates. The standard deviation did not exceed 10% of the mean value.

Fig. 4 shows that, although the maximum activation levels varied among the three different agonists, the levels required to stimulate the T cells were much lower for Ig-PLP1 than for either free PLP1 or native PLP. That is, it took substantially less Ig-PLP1 to stimulate the cell line than either the native PLP or the free peptide (on the order of 1/100). Specifically, stimulation to half the maximum level required less Ig-PLP1 (0.005 μM) than PLP (0.5 μM) or PLP1 peptide (0.8 μM). These results indicate that the PLP1 T cell epitope is better presented by Ig-PLP1 than by native PLP or by synthetic PLP1 peptide. Although the plateau of IL-2 production was higher when the T cell activator is free PLP1 synthetic peptide it requires substantially higher agonist levels that may be difficult to obtain *in vivo* over an extended period.

While not limiting the present invention in any way, it appears that the efficacy of Ig-PLP1 in peptide delivery is related to FcR mediated internalization and access to newly synthesized MHC molecules. More particularly, native PLP appears to internalize rather ineffectively by simple fluid phase pinocytosis while free PLP1 peptide appears to simply bind to empty MHC class II molecules at the cell surface. The ineffectuel presentation of these forms of the autoantigen is clearly illustrated by Fig. 4 which unambiguously shows that Ig-PLP1 is more efficient in presenting PLP1 peptide in combination with MHC class II molecules than either the free peptide or the native protein.

Example VIII

Inhibition of T Cell Activation *in vitro*

Antagonism of PLP1, PLP, and Ig-PLP1 T cell activation by Ig-PLP-LR was detected using a prepulsed proliferation assay.

Irradiated (3,000 rads) SJL splenocytes (used as APCs) were incubated in 96-well round bottom plates (5 x 10^5 cells/well/50 μl) with the selected agonist (1 μM PLP1 peptide, 0.05 μM Ig-PLP1 or 7 μM PLP) and various concentrations of antagonist (100 μl/well) for 1 hour. Subsequently, 4E3 T cell hybridomas (5 X 10^4 cells/well/50 μl) were added and the culture was continued overnight. IL-2 production in the supernatant, determined as in Example VI using HT-2 cells, was used as measure of T cell activation. The results of this assay are shown in Figure 5.

More particularly, Figures 5A, 5B and 5C show antagonism of free PLP1 peptide (5A), Ig-PLP1 chimeric immunoglobulin (5B) and native PLP (5C) respectively. The antagonists were Ig-PLP-LR (squares) and PLP-LR (circles) with controls of Ig-W (diamonds) and PLP2 (triangles).

Cpm values obtained when the APCs were incubated with the agonist but no antagonist was used as control thymidine incorporation. This value was 7,503 ± 1,302 for Ig-PLP1; 31,088 ± 3,860 for PLP1 peptide; and 8,268 ± 915 for PLP. The cpm value obtained when the APCs were incubated with no agonist or antagonist was used as background (BG). This value was 1,560 ± 323 for Ig-PLP1; 2,574 ± 290 for PLP1 peptide; and 2,127 ± 177 for PLP. The percent control thymidine incorporation was calculated as follows: [(cpm obtained in the presence of test antagonist) - (BG)] / [(cpm control thymidine incorporation value) - (BG)]. Each point represents the mean of triplicates.

As previously discussed, the potency of Ig-PLP1 chimeras in peptide loading onto MHC class II molecules may resemble *in vivo* autoimmune circumstances where a continuous supply of antigen often allows for abundant generation of self peptides which can trigger T cell aggressively. Figure 5A (PLP1 agonist) shows that when T cells were incubated with APCs in the presence of both PLP1 and Ig-PLP-LR, a substantial decrease in IL-2 production occurred as the concentration of Ig-PLP-LR increased. A similar decline in IL-2 production was evident when the synthetic PLP-LR peptide was used during T cell activation with PLP1 peptide. Conversely, antagonistic effects were not observed with the control Ig-W immunoglobulin and the PLP2 peptide. Inhibition of IL-2 production to half the maximum level (60% control thymidine incorporation) required only 0.4 μM Ig-PLP-LR versus 9 μM PLP-LR peptide indicating a much more efficient presentation of, and T cell antagonism by, Ig-PLP-LR.

Further evidence that the chimeric immunoglobulin is more efficient than the free peptide in T cell antagonism is shown in Figs. 5B and 5C. Specifically, Fig. 5B shows that Ig-PLP-LR inhibited T cell activation mediated by Ig-PLP1 while free PLP-LR, like the negative control PLP2 peptide, did not show any significant antagonism. Significantly, Fig. 5B also shows that Ig-W, the wild type 91A3 immunoglobulin without any exogenous peptide exhibits partial inhibitory activity in Ig-PLP1 mediated T cell activation. It is believed that this may be the result of competition for binding to the FcR on the APCs because both Ig-PLP1 and Ig-W share identical IgG2b constant regions. A maximum of 50% inhibition in IL-2 production was seen when the activation of T cells by Ig-

PLP1 was carried out in the presence of Ig-W. Thus, Ig-W would compete with Ig-PLP1 for FcR binding and internalization thereby diminishing the activation of T cells. That is, as the concentration of Ig-W increases, less Ig-PLP1 will bind to FcR and be internalize by the APCs resulting in a diminished presentation and corresponding IL-2 production. It is important to note that this Ig-W mediated reduction in response is not the result of antagonistic effects but rather simply a result of competition for FcR binding. That is, the presented Ig-W epitopes are not TCR antagonists for PLP1 and do not interact with the PLP1 specific TCRs.

In contrast to Fig. 5B, Fig. 5C shows that Ig-PLP-LR, but not Ig-W, significantly reduces the activation of T cells by native PLP. As Ig-W is likely internalized in a different manner than native PLP, (Fc receptor versus simple fluid phase pinocytosis) there should not be any direct competition for uptake and processing and hence no inhibition.

For the sake of convenience the results shown in Figure 5 are summarized in Table 1 immediately below. When APCs were incubated with PLP1 peptide in the presence of Ig-PLP-LR there was no activation of the PLP1-specific T cell hybridomas (Figure 5a). Moreover, when the activation of T cells by native PLP and Ig-PLP1 was carried out in the presence of various concentrations of Ig-PLP-LR, IL-2 production (i.e. T-cell activation) declined as Ig-PLP-LR increased. However, free PLP-LR peptide failed to inhibit T cell activation mediated by native PLP or Ig-PLP1. These two lines of evidence indicate that the principal mechanism for Ig-PLP-LR mediated inactivation of T cells was likely to be endocytic presentation and TCR antagonism rather than direct blockage of MHC class II molecules on the cell surface.

In the table below a plus sign indicates inhibition of IL-2 production and therefore antagonism, while a minus sign indicates little or no inhibition of IL-2 production and therefore little or no antagonism.

Table 1.

Ig-PLP-LR and PLP-LR Mediated T Cell Antagonism.

	Stimulator (Agonist)		
Antagonist	**PLP1**	**PLP**	**Ig-PLP1**
PLP-LR	+	-	-
Ig-PLP-LR	+	+	+

The results of the foregoing example indicate that the FcR mediated uptake and subsequent processing of a peptide antagonist are compatible with efficient presentation by the antigen presenting cell. This is extremely

unexpected in view of the prior art where the delivery of free peptide analogs was assumed to provide efficient antagonism through direct competition for MHC or TCR binding sites.

Example IX

Characterization of Mechanism for Antagonism by Ig-PLP-LR

Using an assay similar to the one performed in Example VIII, it was demonstrated that competition for direct binding to the Fc receptor is not, in and of itself, a likely mechanism for Ig-PLP-LR mediated antagonism.

SJL splenic APCs were incubated with native PLP (6.8 μM) in the presence of 2 μM Ig-PLP2, Ig-PLP-LR, or Ig-W and assayed for IL-2 production by ^{3}H-thymidine incorporation using HT-2 cells as described in the previous Examples. Ig-PLP2 was prepared as in Example II using the sequence detailed in Example I. The % control thymidine incorporation was calculated as in Example VIII. Results of the assay are shown in Fig. 6 wherein each column represents the mean ± SD of triplicates.

As with the results shown in Fig. 5B, the present Example supports the position that both efficient presentation on the MHC class II structure and an effective peptide analog provide the most significant results. That is, even though the Ig-PLP2 chimeric antibody is taken up and processed, efficient presentation of the PLP2 peptide by I-A^s will not preclude activation of the T-cells as it is not an analog of the native PLP agonist. Accordingly, simple competition binding to MHC class II molecules on the antigen presenting cells is not likely to produce the desire antagonism.

Example X

In vivo Induction of a T Cell Response to PLP1

By this Example it was demonstrated that, in addition to generating a T cell response *in vitro* (Example VII), the chimeric antibodies of the present invention could be used to generate a cellular response *in vivo*. Specifically, the following Example demonstrates the *in vivo* priming of PLP1 specific T cells by Ig-PLP1.

Six to eight week old SJL mice (H-2^s) were purchased from Harlan Sprague Dawley (Frederick, MD) and maintained in an animal facility for the duration of experiments.

The mice were immunized subcutaneously in the foot pads and at the base of the limbs and tail with 50 μg of Ig-PLP1 emulsified in a 200 μl mixture of 1:1 v/v PBS/CFA. Ten days later the mice were sacrificed by cervical dislocation, the spleens and lymph nodes (axillary, inguinal, popliteal, and sacral) were removed, single cell suspension were prepared, and the T cell responses were analyzed. The results shown in Figure 7 are those obtained with 4 x 10^5 lymph node cells/well (7A) and 10 x 10^5 spleen cells/well (7B). The activators PLP1 and PLP2 were used at 15 μg/mL and PPD was used at 5 μg/mL.

As with the previous Examples, T cell activation was monitored using a proliferation assay comprising ^{3}H-thymidine incorporation. Here, lymph node and spleen cells were incubated for three days in 96-well round bottom plates, along with 100 μl of a single selected activator, at 4 and 10 x 10^5 cells/100 μl/well, respectively. Subsequently, 1 μCi ^{3}H-thymidine was added per well, and the culture was continued for an additional 12-14 hours.

The cells were then harvested on glass fiber filters, and incorporated ^{3}H-thymidine was counted using the trace 96 program and an Inotech β counter. A control media with no stimulator was included for each mouse and used as background.

Each value shown in Figure 7 was calculated as described in Example VIII and represents the mean ± SD of triplicates after deduction of background cpms obtained with no activator in the media. Similar results were obtained when mice were immunized with 150 μg of Ig-PLP per mouse (not shown).

Figures 7A and 7B clearly show that, when Ig-PLP1 was injected subcutaneously in the foot pads and at the base of the limbs and tail, a strong specific T cell response to the PLP1 peptide was induced. While there was some variation as to the strength of the reaction among the individual mice, the lymph node and spleen cells of each produced a significant response upon challenge with the PLP1 peptide. Interestingly there is a significant PLP1 specific response detected in the spleen, an organ that mostly filters and responds to systemic antigens. One possibility that can be put forth to explain these results is that Ig-PLP1, because of it's long half life, was able to circulate and reach both the lymphatic and blood circulation and consequently be presented at both systemic and lymphatic sites. This is potentially very beneficial when implementing therapeutic regimens for autoimmune disorders. It was also interesting that some mice show proliferation when the cells are stimulated with PLP2 peptide *in vitro*. Possibly, the fact that this peptide is presented by I-A^s like PLP1 allows low affinity cells to bind and generate a response. In any case the results are consistent with those provided by the earlier Examples where it was shown that Ig-PLP1 was efficient in presenting the peptide to T cells *in vitro*.

Example XI

In vivo Inhibition of a T Cell Response to PLP1

As seen in the previous Example, Ig-PLP1 is capable of priming T cells *in vivo* and generates a potent immune response when exposed to the agonist PLP1 peptide. This Example demonstrates that the administration of a peptide antagonist in the form of a chimeric antibody immunomodulating agent can substantially reduce the immune response generated by the endocytic presentation of an agonist ligand. Specifically, this Example demonstrates that co-administration of Ig-PLP-LR with Ig-PLP1 significantly reduces the immune response to PLP1 peptide.

Mice were co-immunized with mixtures of either 50 μg Ig-PLP1 and 150 μg Ig-PLP-LR or 50 μg Ig-PLP1 combined with 150 μg Ig-W. In particular, individual mice from three groups (4 mice per group) were injected sc. as in Example X with a 200 μl mixture (PBS/CFA, 1:1 v/v) containing one of the following mixtures: 50 μg Ig-PLP1 and 150 μg Ig-PLP-LR; 50 μg Ig-PLP1 and 150 μg Ig-W; or Ig-PLP1 and 100 μg PLP-LR peptide. Splenic and lymph node T cell responses were analyzed at day 10 post immunization using the protocol set forth in Example X. The lymph node cells were assayed at 4 x 10^5 cells/well and the spleen cells at 10 x 10^5 cells/well. The agonist ligand was PLP1 at 15 μg/mL. Results for the lymph node and spleen cells, shown in Figs. 8A and 8B respectively and summarized in Table 2 below, represent the mean ± SD of triplicates after deduction of background cpm obtained with no agonist in the media.

Figures 8A and 8B show that, although Ig-PLP1 was efficiently presented and induced a strong *in vivo* T cell response (Example X), it was possible to antagonize such a response by including Ig-PLP-LR in the mixture administered to mice. Indeed, when Ig-PLP1 was co-administered to mice with Ig-PLP-LR, the subsequent immune response to free PLP1 peptide was markedly reduced as shown on the right half of Figs. 8A and 8B. It appears that the low PLP1 response for both the spleen and lymph node tissue was a result of PLP-LR antagonism, since the co-administration with Ig-PLP1 of the wild type antibody, Ig-W, did not significantly reduce the T cell response. These results strongly indicate that it is the efficient *in vivo* presentation of PLP-LR through the FcR binding and endocytic processing of Ig-PLP-LR that is responsible for the reduced cellular response.

Moreover, as seen in Table 2 immediately below, when free PLP-LR peptide was co-administered with Ig-PLP1 there was no indication that the PLP1 response was reduced. The numbers provided in the table represent the percentage values of PLP1 specific proliferation relative to PPD specific proliferation and were derived as follows: (mean cpm of triplicates obtained with PLP1 stimulation - mean cpm triplicate BG) /(mean cpm of triplicates obtained with PPD - mean cpm triplicate BG) x 100

Table 2

Ig-PLP-LR But Not Free PLP-LR Peptide Mediates T Cell Antagonism In Vitro

	Ig-PLP1 co-administered with:		
Mouse	Ig-W	Ig-PLP-LR	PLP-LR peptide
	PLP1/PPD (%)		
1	100	28	81
2	95	40	91
3	78	37	93
4	79	25	100

The results above clearly show that co-administration of the free antagonist peptide or the control Ig-W lacking an antagonist peptide have little effect on the generated immune response. The lack of antagonist effect by free PLP-LR peptide was not due to a net lower amount of injected peptide because the mice were given approximately 34 fold more PLP-LR in the free peptide form than in the Ig-PLPLR form (on the basis of a MW of 150,000 D, the 150 μg of Ig-PLP-LR given to the mice correspond to 1 nmole of Ig that contains 2 nmoles of PLP-LR peptide, while with a MW of 1,468 Daltons the 100 μg of free PLP-LR peptide corresponds to 68 nmoles of

peptide). The failure of PLP-LR peptide to inhibit Ig-PLP1 mediated T cell activation coupled with the potency of Ig-PLP-LR in antagonizing Ig-PLP1 T cell stimulation supports the belief that Ig-PLP-LR mediated *in vivo* antagonism is likely related to efficient presentation.

Example XII

Induction of a T Cell Response to an Endocytically Presented Antagonist

Previous Examples have shown that administration of chimeric antibodies comprising a agonist ligand can prime immune cells *in vivo*. It was also shown that administration of a chimeric antibody comprising an antagonist can reduce a subsequent response to challenge by an agonist ligand. This Example demonstrates that efficient presentation of an antagonist can prime immune cells *in vivo* and mount a strong response that could effect the reaction of the T cells to an agonist peptide. Specifically, mice co-injected with Ig-PLP1 and Ig-PLP-LR develop a relatively high proliferative response to PLP-LR and practically no response to PLP1 peptide.

Lymph node and spleen cells were obtained in the same manner as set forth in Example X following co-administration of Ig-PLP1 and Ig-PLP-LR. Proliferative responses in individual mice were also measured using the methods set out in the previous Example following in vitro stimulation with either free PLP1 peptide or PLP-LR peptide at 15 μg/mL. The results of the assays using lymph node and spleen cells are detailed in Figures 9A and 9B respectively.

As can be seen from Figure 9, both spleen and lymph nodes developed responses to the antagonist PLP-LR but not to the PLP agonist PLP1. Knowing that Ig-PLP-LR induced PLP-LR specific T cells when it was co-administered with Ig-PLP1, it can be speculated that these PLP-LR-specific T cells downregulate PLP1 specific T cells. Conversely, although there was induction of PLP-LR-specific response when free PLP-LR peptide was administered with Ig-PLP1 (not shown), there was no evident reduction in the proliferative response to PLP1. Accordingly, the data set forth in the instant example demonstrates that the use of chimeric antibodies comprising an antagonist are much more effective for modulating the immune response to an antigen agonist than the free peptide antagonist.

More particularly, in view of the foregoing examples it appears that TCR engagement with PLP-LR-I-A^S complexes (i.e. MHC-PLP-LR complexes) on the surface of APCs antagonizes T cells rather than stimulates them. Accordingly, antagonism by Ig-PLP-LR may occur because efficient presentation of Ig-PLP-LR in endocytic vacuoles ensures significant levels of PLP-LR-I-A^S complexes (antagonist complexes) are generated. The amount of complexes on the cell surface is proportional to the amount of Ig-PLP-LR offered to the APCs. When PLP1 stimulation is carried out in the presence of Ig-PLP-LR, both PLP-LR-I-A^S and PLP1-I-A^S are present on the surface of a given APC where an increase in the concentration of Ig-PLP-LR leads to higher number of PLP-LR-I-A^S complexes. It will be appreciated that approximately 3500 TCR have to be engaged in order for a T cell to be activated and that a given complex of MHC class II-peptide complex serially engages approximately 200 TCRs. As such, it appears that a T cell is antagonized when TCR engagement with PLP-LR-I-A^S complexes override engagement with the agonist PLP1-I-A^S. Overall, because of efficient loading of PLP-LR by Ig-PLP-LR, T cell antagonism is achieved by a higher frequency of serial triggering of TCR by PLP-LR-I-A^S complexes. That is, the efficient uptake and processing of Ig-PLP-LR simply

means that too many of the surface MHC complexes present the PLP-LR antagonist to allow the remaining surface complexes presenting the PLP1 agonist ligand to engage the number of TCRs to activate the T cell. Therefore, the T cells will not be activated as long as the antagonist is presented at a rate that ensures the activation concentration of MHC class II-agonist complexes is not reached on the APC.

Example XIII

Lymph Node Proliferative Responses to Immunization With Ig-PLP Chimeras

Proliferative responses were measured in mice immunized with individual Ig-PLP chimeras or varying mixtures of Ig-PLP1 and Ig-PLP-LR. It was observed that Ig-PLP-LR given alone to mice induced T cells which, like those induced by Ig-PLP1, cross-reacted with both PLP1 and PLP-LR peptides. Surprisingly, however, despite the cross-reactivity of the responses, when the chimeras were administered together they displayed a dose dependent antagonism on one another resulting in down-regulation of both T cell responses. Finally, antigen specific T cells induced either by IG-PLP 1 or by IG-PLP-LR were refractory to down-regulation by peptide mixtures and proliferated significantly when they were in vitro stimulated simultaneously with both PLP1 and PLP-LR. These findings indicate that both agonist and antagonist peptides exert adverse reactions on one another and reveal an anti-parallel antagonism and a stringent control of TCR triggering at the level of naive T cells.

Materials were obtained and mice immunized as described above. Proliferative responses were measured by thymadine incorporation as set forth in Example VI above. Lymph node and spleen cells were obtained in the same manner as set forth in Example X following co-administration of Ig-PLP1 and Ig-PLP-LR. Mice were injected with 50 μg Ig-PLPI (10A), 50 μg Ig-PLP-LR (10B), 100 μg PLP1 (10C) or 100 μg PLP-LR (10D) in CFA, and 10 days later the lymph node cells were in vitro stimulated with the indicated free peptides. The stimulators PLP1, PLP-LR and PLP2 were used at the defined optimal concentration of 15 μg/ml.

The data illustrated in figs 10A-10D indicate that Ig-PLP1, like PLP1 peptide, induced a specific T cell response to PLP1 peptide. Similarly, Ig-PLP-LR, like PLP-LR peptide, induced a specific T cell response to PLP-LR peptide. Neither the Ig chimera nor the free peptides induced T cells that significantly reacted with the negative control PLP2, a peptide that is also presented by I-A^s class II molecules. Surprisingly, however, the response induced by Ig-PLP1 cross-reacted with PLP-LR peptide, while the response induced by Ig-PLP-LR cross-reacted with PLP1. The responses induced with free PLP1 or free PLP-LR were not cross-reactive.

Example XIV

Lymph Node T cell Proliferative Response to
Co-Immunization With Ig-PLP1 and Ig-PLP-LR

Mice were injected with the indicated chimeras and 10 days later the lymph nodes cells were in vitro stimulated with free peptides, and assayed for proliferation by [^{3}H]thymidine incorporation as detailed above. The results are shown in Fig. 11.

The number preceding the Ig chimera label indicates the μg amount injected per mouse. The stimulators were PPD, 5 μg/ml; PLP 1, PLP-LR, and PLP2 at 15 μg/ml. Cells incubated without stimulator were used as background (BG). The mice were tested individually and triplicate wells were assayed for each stimulator. To standardize the results and eliminate intrinsic individual variability we expressed the results as relative proliferation estimated as follows: (mean test peptide cpm - mean BG cpm)/(mean PPD cpm - mean BG cpm). The indicated relative proliferation represents the mean ± SD of 5 mice tested individually. The mean cpms ± SD obtained with PPD stimulation for the different groups of mice were as follows: 50μg Ig-PLP1:16,413 ± 1330; 50μg Ig-PLP-LR: 11,224 ± 3481; 50μg Ig-W: 11,513 ± 1,572; 50μg Ig-PLP1 + 50μg Ig-PLP-LR: 16,817 ± 2,869; 50μg Ig-PLP1 + 150μg Ig-PLP-LR: 16,156 ± 2006; 50μg Ig-PLP1 + 150μg Ig-W: 11,699 ± 1,142; 50μg Ig-PLP-LR + 150μg Ig-W: 13,435 ± 1,650; 50μg Ig-PLP1 + 50μg Ig-PLP2: 10,056 ± 1,407; and 50μg Ig-PLP-LR + 50μg Ig-PLP2: 10,877 ± 563. Filled and hatched bars indicate proliferation to PLP1 and PLP-LR respectively. The proliferation to PLP2 peptide was at background levels except where Ig-PLP2 was used in the immunization mixture.

As can be seen in Figure 11, lymph node T cells from a group of mice that were immunized with Ig-PLP1 proliferated equally well to PLP1 and to PLP-LR whereas Ig-W control caused little reaction. Surprisingly, the PLP-LR response was at background levels. Accordingly, although the responses to the Ig chimeras share cross-reactivity between PLPL and PLP-LR peptides, the mixture yielded down regulation rather than additive responses. In fact, the data suggest an anti-parallel down regulation among Ig-PLP1 (agonist) and Ig-PLP-LR (antagonist). This down-regulation appeared to be dose dependent because mice that were injected with a mixture of 50 μg Ig-PLP1 and 150 μg Ig-PLP-LR failed to respond to PLPI and mounted responses to PLP-LR that were reduced to levels observed with mice injected with Ig-PLP1 alone.

One possible explanation for the observed opposite down regulation between IG-PLPI and Ig-PLP-LR is that clonal expansion requires an optimal serial triggering with an homogeneous peptide (i.e. all or most of the receptors on a single naive T cell must engage one type of peptide in order to expand). Simultaneous stimulation of naive t cells with peptides encompassing subtle differences at the TCR contact residues, which may be occurring during immunizations involving mixtures of Ig-PLP1 and Ig-PLP-LR, fails to cause T cell expansion and in vitro proliferation.

Example XV

Splenic Proliferative T Cell Responses of Mice
Co-Immunized with Ig-PLP1 and IG-PLP-LR

As shown in Figure 12, spleen cells from the mice described in Example XIV were stimulated with PLP1 (filled bars) and PLP-LR (hatched bars.) in triplicate wells and proliferation was measured as above. The results were standardized as above using PPD cpms obtained with lymph node T cells because the proliferation of spleen cells upon stimulation with PPD was minimal. The indicated relative proliferation represents the meant ± SD of 5 individually tested mice.

Splenic T cells from these mice failed to respond to PLP-LR stimulation. However, when an additional group of mice was immunized with Ig-PLP-LR, both lymph node and splenic cells proliferated to PLP1 as well as to PLP-LR

peptide. In the spleen, although the proliferative responses were much lower than in the lymph nodes, additive responses were still not observed. Rather, an opposite down-regulatory effect between Ig-PLP1 and Ig-PLP-LR was observed. Although co-injection of Ig-W with either Ig-PLP1 or Ig-PLP-LR did not affect either response, co-injection of Ig-PLP2 with Ig-PLP1 increased reactivity to PLP-LR among the T cells induced by Ig-PLP1.

Example XVI

IL-2 Production by Splenic Cells of Mice

Co-Immunized With Ig-PLP1 and Ig-PLP-LR

To further investigate the opposing down regulation among Ig-PLP1 and Ig-PLP-LR, splenic antigen induced cytokine responses were measured in animals immunized with either a single or both Ig-chimeras. As shown in Fig. 13, spleen cells (1 X 10^6 per well) from the mice described in Example XIV were stimulated with PLP1 (filled bars) and PLP-LR (hatched bars) for 24 hours. Production of IL-2 (13A), INFγ (13B), and IL-4 (13C) were measured as set forth below.

Cells were incubated in 96 well round-bottom plates at 10 x 10^5 cells/100μl/well with 100μl of stimulator, as above, for 24 hours. Cytokine production was measured by ELISA according to Pharmingen's instructions using 100μl culture supenatant. Capture antibodies were rat anti-mouse IL-2, JES6-1A12;rat anti-mouse IL-4, 11B11;rat anti-mouse IFNγ, R4-6A2; and rat anti-mouse IL10, JES5-2A5. Biotinylated anti-cytokine antibodies were rat anti-mouse IL-2, JES6-5H4; rat ant imouse IL-4, BVD6-24G2; rat anti-mouse IFNγ, XMG 12; and rat anti-mouse IL- 10, JES5-16E3) The OD405 was measured on a Spec 340 counter (Molecular Devices) using SOH MAX PRO version 1.2.0 software. Graded amounts of recombinant mouse IL-2, IL-4, INFγ, and IL-10 were included in all experiments in order to construct standard curves. The concentration of cytokines in culture supernatants was estimated by extrapolation from the linear portion of the standard curve. Cells incubated without stimulator were used as background (BG). Each mouse was individually tested in triplicate wells for each stimulator and the indicated cpms represent the mean ± SD after deduction of BG cpms. Production of IL-10 was also measured, but the results were at background levels (not shown).

Upon in vitro stimulation with PLP1 peptide, T cells from Ig-PLP1 immunized mice produced IL-2, INFγ, and small amounts of IL-4. However, stimulation of the same cells with PLP-LR yielded minimal IL-2 and undetectable INFγ or IL-4. Spleen cells from Ig-PLP-LR immunized mice generated IL-2 but no IFNγ or IL-4 upon stimulation with PLP1 peptide. Moreover, PLP-LR peptide stimulation produced only a minimal IL-2 response. In mice immunized with equal amounts of Ig-PLP1 and Ig-PLP-LR all cytokine production was reduced to minimal or background levels upon stimulation with either peptide. Co-immunization of Ig-W with either chimera had no measurable effect on cytokine production pattern. When the animals were given a 3:1 ratio of Ig-PLP-LR: Ig-PLP1, although the splenic proliferative responses and IL-2 production were at background levels, significant amounts of IL-4 and INFγ were evident upon stimulation with PLP-LR peptide. Consequently, the excess of Ig-PLP-LR may lead to a mixed but PLP-LR dominant TCR triggering that induces cells able to produce cytokine but which exhibit no proliferative response. These data

indicated that Ig-PLP1 and Ig-PLP-LR exerted adverse reactions on one another leading to down-regulation of both T cell responses.

Example XVII

Proliferation of Antigen Experienced T Cells Upon
Stimulation In Vitro With Mixtures of PLPI and PLP-LR Peptides

To investigate whether Ig-PLP1 and Ig-PLP-LR could display adverse reactions on each other at the level of antigen experienced cross-reactive T cells, mice were immunized with Ig-PLP1 or Ig-PLP-LR alone and assessed for proliferative T cell responses upon *in vitro* stimulation with varying mixtures of free PLPI and PLP-LR peptides.

More particularly Mice (4 per group) were immunized with 50μg Ig-PLP1 (14A and 14B) or 50μg Ig-PLP-LR (14C and 14D) in CFA, and 10 days later the lymph node (14A and 14C) and spleen (14B and 14D) cells were stimulated with the indicated peptides and assayed for [^{3}H]thymidine incorporation as above. The number preceding the peptide label indicates the μg/ml amount used for in vitro stimulation. The specific proliferation was estimated by deducting the mean BG (obtained by incubating cells without stimulator) cpm from the test sample cpm. The indicated cpms represent the mean ± SD of 4 individually tested mice. ND, not determined.

As can be seen in Figs. 14A-14D, both lymph node and spleen cells from mice immunized with Ig-PLPI or Ig-PLP-LR proliferated equally as well to stimulation with a single peptide as to a mixture of PLPL and PLP-LR. The proliferative response to the mixture,in most cases, was even higher than the response to a single peptide stimulation.

Example XVIII

IL-2 Production by Antigen Experienced T Cells
Upon In Vitro Stimulation With PLP1/PLP-LR Peptide Mixtures

To further investigate whether Ig-PLP1 and Ig-PLP-LR could display adverse reactions on each other at the level of antigen experienced cross-reactive T cells, mice were immunized with Ig-PLP1 or Ig-PLP-LR alone and assessed for cytokine responses upon *in vitro* stimulation with varying mixtures of free PLPI and PLP-LR peptides. The results are shown in Figs. 15A and 15B.

Spleen cells from Ig-PLP1 (15A) and Ig-PLP-LR (15B) immunized mice were stimulated with the indicated peptides and tested for IL-2 production by ELISA as in Example XVI. The spleen cells used in these experiments were from the mice described in Example XVII. The number preceding the name of the peptide represents the μg/mi amount used for stimulation. The indicated μg/ml IL-2 values represent the mean d: SD of 4 individually tested mice.

As indicated by Example XVII, IL-2 production was not decreased upon stimulation of spleen cells with varying mixtures of PLP1 and PLP-LR. To the contrary, in most cases of stimulation with peptide mixture IL-2 production was higher than in stimulation with a single peptide. Again these findings indicate that both agonist and antagonist peptides exert adverse reactions on one another and reveal an anti-parallel antagonism and a stringent control of TCR triggering at the level of naive T cells.

In addition to the use of immunomodulating agents comprising T cell receptor antagonists and agonists for attenuation of adult immune responses, the same compositions may advantageously be used for the induction of tolerance in neonates and infants as demonstrated in the following Examples.

5

Example XIX

Ig-PLP1 Injected with Ig-PLP1 at Birth

Resist Induction of EAE During Adult Life

To demonstrate the advantages of inoculating neonates or infants with the compositions of the present invention, newborn mice were administered immunomodulating agents as described herein and exposed to agents for the inducement of an autoimmune condition.

More specifically, neonatal mice (10 mice per group) were injected with 100 μg of affinity chromatography purified Ig-PLP1 or Ig-W within 24 hours of birth and were induced for EAE with free PLP1 peptide at 7 weeks of age. Mice were scored daily for clinical signs as follows: 0, no clinical signs; 1, loss of tail tone; 2 , hind limb weakness; 3, hind limb paralysis; 4, forelimb paralysis; and 5, moribund or death. Panel A shows the mean clinical score of all mice and panel B shows the mean score of the surviving animals only. EAE was induced by subcutaneous injection in the foot pads and at the base of the limbs and tail with a 200 μl IFA/PBS (1vol/1vol) solution containing 100 μg free PLP1 peptide and 200 μg *M. tuberculosis* H37Ra. Six hours later 5 x 10^8 inactivated B. *pertussis* were given intravenously. After 48 hours another 5 x 10^8 inactivated *B. pertussis* were given to the mice.

As may be seen in Figs 16A and 16B adult mice recipient of Ig-PLP1 in saline at birth resisted the induction of EAE by free PLP1 peptide. Indeed, the clinical scores were much less severe in those mice than in animals recipient of Ig-W, the parental wild type Ig without any PLP peptide. In addition, contrary to those mice which received Ig-W, mice injected with Ig-PLP1 showed no relapses (figure 16B).

Example XX

In Vivo Presentation of Ig-PLP1 by Neonatal

Thymic and Splenic Antigen Presenting Cells

In order to confirm the clinical results observed in Example XX, cytokine responses were measured in neonatal mice. The data obtained is shown in Fig. 17.

Specifically, neonates (5 mice per group) were injected with 100 μg Ig-PLP1 or Ig-W within 24 hours of birth. Two days later the mice were sacrificed, and pooled thymic (17A) and splenic (17B) cells were irradiated and used as APCs for stimulation of the PLP1-specific T cell hybridoma 4E3 as described above. IL-2 production in the supernatant which was used as a measure of T cell activation was determined using the IL-2 dependent HT-2 cell line as described by V.K. Kuchroo et al. *J. Immunol.* 153, 3326 (1994) incorporated herein by reference. The indicated cpms represent the mean ± SD of triplicates.

The administered Ig-PLP1 was efficiently presented by neonatal APCs. Both thymic (17A) and splenic (17B) APCs from neonate recipients of IG-PLP1 activated a T cell hybridoma specific for PLP1 peptide without addition of erogenous antigen. APCs from neonate recipients of Ig-W were unable to activate the T cell hybridoma.

Example XXI

Reduced Splenic Proliferative T cell

Response in Mice Recipient of Ig-PLP1 at Birth

To further confirm the results observed in the previous two Examples, proliferative responses were measured in mice inoculated with an immunomodulating agent at birth. The results are shown in Figs. 18A and 18B.

Neonates were injected intraperitoneal (i.p.)within 24 hours of birth with 100 μg Ig-PLP1 or Ig-W in saline. When the mice reached 7 weeks of age they were immunized with 100 μg free PLP1 peptide in 200 μl CFA/PBS (1vol/1vol) s.c. in the foot pads and at the base of the limbs and tail. Ten days later the mice were sacrificed, and (18A) the lymph node (0.4 x 10^6 cells/well) and (18B) the splenic (1 X 10^6 cells/well) cells were in vitro stimulated for four days with 15 μg/ml free PLP1 or PLP2, a negative control peptide corresponding the encephalitogenic sequence 178-191 of PLP (13). One μCi/well of[^{3}H]thymidine was added during the last 14.5 hours of stimulation, and proliferation was measured using an Inotech β-counter and the trace 96 Inotech program. The indicated cpms represent the mean ± SD of triplicate wells for individually tested mice. The mean cpm ± SD of lymph node proliferative response of all mice recipient of Ig-PLP1 and Ig-W was 34,812 ± 7,508 and 37,026 ± 10,133, respectively. The mean splenic proliferative response was 3,300 ± 3,400 for the Ig-PLP1 recipient group and 14,892 ± 4,769 for the Ig-W recipient group.

Mice recipient of Ig-PLP1 at the day of birth, like those injected with Ig-W, developed equivalent adult lymph node T cell proliferative responses to PLP1 when they were immunized with free PLP1 peptide in CFA (18A). However, the splenic proliferative response was markedly reduced in the mice recipient of Ig-PLP1 (18B) thus indicating the inducement of tolerance. Neither group of mice showed a significant proliferative response to PLP2, a negative control peptide presented by I-A^s class II molecules like PLP1.

Example XXII

Lymph Node T Cell Deviation in Mice Treated With Ig-PLP1 at Birth

To further demonstrate the induction of tolerance in infants or neonates, cytokine responses were measured in were measured in mice inoculated with an immunomodulating agent at birth. The results are shown in Figs. 19A-19C.

In particular, lymph node cells (4 x 10^6 cells/well) from the mice described in Example XXI were stimulated *in vitro* with free PLP1 or PLP2 (15 μg/ml) for 24 hours, and the production of IL-2 (19A), IL-4 (19B), and IN$F\gamma$ (19C) was measured by ELISPOT as described in Example XVI using Pharmingen anti-cytokine antibody pairs. The indicated values (spot forming units) represent the mean ± SD of 8 individually tested mice.

The results show cytokine production patterns were affected by the inoculation of the neonatal mice. Lymph node cells from mice recipient of Ig-W at birth produced, upon stimulation with PLP1, IL-2 but not IN$F\gamma$ or IL-4. In contrast, cells from mice recipient of Ig-PLP1 were deviated and instead produced IL-4. No cytokine production was observed upon stimulation with PLP2 peptide.

Example XXIII

Reduced INFγ Production by Splenic T Cells From
Mice Injected With Ig-PLP1 at the Day of Birth

To confirm the results obtained in Example XXII, spleen cells from the same mice were assayed for cytokine responses. The results are shown in Figs. 20A and 20B.

More specifically, splenic cells (1 X 10^6 cells/well) from the mice were stimulated *in vitro* with free PLP1 or PLP2 (15 μg/ml) for 24 hours, and the production of IL-2 (20A), IL-4 (20B), and INFγ (20C) in the supernatant was measured by ELISA using pairs of anti-cytokine antibodies from Pharmingen according to the manufacture's instructions (Example XVI). The indicated amounts of cytokine represent the mean ± SD of 8 individually tested mice.

In the spleen, while cells from mice innoculate with Ig-W produced IL-2 and INFγ. Conversely, cells from mice injected with Ig-PLP1 produced IL-2 but failed to produce detectable levels of INFγ. The negative control, PLP2 peptide, failed to induce cytokine production.

Example XXIV

Cytokine Mediated Restoration of Splenic T Cell
Proliferation in Mice Injected With Ig-PLP1 at Birth

To demonstrate that proliferative responses may be retstored, cells from inoculated neonatal mice were exposed to exogonous INFγ. The results are shown in Fig. 21.

In particular, a group of neonates injected i.p. with 100 μg of Ig-PLP1 at birth were immunized with 100 μg PLP1 peptide in CFA, as in Example XXI, and *in vitro* stimulation of splenic cells (1 x 10^6 cells/well) with free PLP1 peptide (15 μg/ml) was carried out as described in Example XXI but in the presence of 100 units INFγ or IL-12. The indicated cpms for each mouse represent the mean ± SD of triplicate wells.

Surprisingly, addition of erogenous INFγ to splenic cells from the mice recipient of Ig-PLP1 at birth restored the proliferative response. IL-12, an inducer of INFγ (14), also restored the splenic proliferative response.

Overall, mice injected at birth with Ig-PLP1 develop a lymph node T cell deviation and an unusual INFγ-mediated splenic anergy. Interestingly, when these mice were induced for EAE with free PLP1 peptide they developed a mild monophasic disease without relapses. Since Igs have long half-lives, an Ig based immunomodulating agent may endure for an extended period of time resulting in a continuous and slow release of the immunosuppressive factor, as may occur in the usual neonatal tolerization procedures using incomplete Freund's adjuvant with a conventional antigen. Consequently, delivery on Igs may allow one to circumvent the use of adjuvant to induce neonatal tolerance. Further, internalization of an immunosuppressive factor via FcR and the subsequent processing in the endocytic pathway grants access to newly synthesized MHC class II molecules, generating significant amounts of MHC-immunosuppressive factor complexes. These favorable parameters (i.e. FcR-mediated APCs activation, slow peptide release, and efficient peptide presentation), may contribute to the induction of lymph node deviation and

splenic anergy. As with administration of the disclosed compositions to adults, the adjuvant free tolerization strategy may be used to silence autoreactive T cells and prevent autoimmunity.

Those skilled in the art will further appreciate that the present invention may be embodied in other specific forms without departing from the spirit or central attributes thereof. In that the foregoing description of the present invention discloses only exemplary embodiments thereof, it is to be understood that other variations are contemplated as being within the scope of the present invention. Accordingly, the present invention is not limited to the particular embodiments which have been described in detail herein. Rather, reference should be made to the appended claims as indicative of the scope and content of the invention.

WHAT IS CLAIMED IS:

1. An immunomodulating agent for the endocytic presentation of an immunosuppressive factor on the surface of an antigen presenting cell of a vertebrate comprising at least one Fc receptor ligand and at least one immunosuppressive factor.

2. The immunomodulating agent of claim 1 wherein said immunosuppressive factor is selected from the group consisting of T cell receptor antagonists, T cell receptor agonists and combinations thereof.

3. The immunomodulating agent of claim 2 wherein said immunosuppressive factor comprises a peptide antagonist.

4. The immunomodulating agent of claim 3 wherein said peptide antagonist is an analog of a peptide agonist capable of activating a T cell response to proteolipid protein.

5. The immunomodulating agent of claim 1 wherein said at least one Fc receptor ligand comprises at least part of a domain of a constant region of an immunoglobulin molecule.

6. The immunomodulating agent of claim 1 wherein the immunomodulating agent comprises a polypeptide.

7. The immunomodulating agent of claim 1 wherein the immunomodulating agent comprises an antibody-antigen complex.

8. The immunomodulating agent of claim 1 wherein the immunomodulating agent is a chimeric antibody.

9. The immunomodulating agent of claim 8 wherein said chimeric antibody comprises a T cell receptor antagonist.

10. The immunomodulating agent of claim 9 wherein said T cell receptor antagonist is expressed within at least one complementarity determining region.

11. A pharmaceutical composition for the endocytic presentation of an immunosuppressive factor on the surface of an antigen presenting cell of a vertebrate comprising a compound as set forth in any one of claims 1-10.

12. Use of an immunomodulating agent as set forth in any one of claims 1-10 for the preparation of a pharmaceutical composition to treat an immune disorder in a patient in need thereof.

13. The method of claim 12 wherein said immune disorder comprises a disorder selected from the group consisting of autoimmune disorders, allergic responses and transplant rejection.

14. The method of claim 13 wherein said immune disorder comprises an autoimmune disorder selected from the group consisting of multiple sclerosis, lupis, rheumatoid arthritis, scleroderma, insulin-dependent diabetes and ulcerative colitis.

15. The method of claim 12 wherein said patient is an infant or neonate.

16. Use of an immunomodulating agent as set forth in any one of claims 1-10 for the preparation of a pharmaceutical composition for the induction of T cell tolerance in a patient in need thereof.

17. The method of claim 16 wherein said T cell tolerance is associated with an autoimmune disorder selected form the group consisting of multiple sclerosis, lupus, rheumatoid arthritis, sclerdoerma, insulin-dependent diabetes and ulcerative colitis.

18. The method of claim 16 wherein said patient is an infant or neonate.

19. A method for treating an immune disorder comprising:

administering to a patient a therapeutically effective amount of a pharmaceutical composition comprising an immunomodulating agent in combination with a physiologically acceptable carrier or diluent wherein said immunomodulating agent comprises at least one Fc receptor ligand and at least one immunosuppressive factor.

20. The method of claim 19 wherein said immunosuppressive factor is selected from the group consisting of a T cell receptor antagonist, a T cell receptor agonist and combinations thereof.

21. The method of claim 19 wherein said immunosuppressive factor is an analog of a peptide agonist capable of activating a T cell response to proteolipid protein.

22. The method of claim 19 wherein said immunosuppressive factor is an analog of a peptide agonist capable of activating a T cell response to myelin basic protein.

23. The method of claim 19 wherein said Fc receptor ligand comprises at least part of one domain of a constant region of an immunoglobulin molecule.

24. The method of claim 23 wherein the immunoglobulin molecule is human IgG molecule.

25. The method of claim 19 wherein said immunomodulating agent comprises a polypeptide.

26. The method of claim 25 wherein said immunomodulating agent comprises a chimeric antibody.

27. The method of claim 19 wherein said immune disorder comprises a disorder selected from the group consisting of autoimmune disorders, allergic responses and transplant rejection.

28. The method of claim 27 wherein said immune disorder comprises an autoimmune disorder selected from the group consisting of multiple sclerosis, lupis, rheumatoid arthritis, scleroderma, insulin-dependent diabetes and ulcerative colitis.

29. A method for producing an immunomodulating agent for the endocytic presentation of an immunosuppressive factor on the surface of an antigen presenting cell of a vertebrate comprising the steps of:

transforming or transfecting suitable host cells with a recombinant polynucleotide molecule comprising a nucleotide sequence which encodes a polypeptide comprising at least one Fc receptor ligand and at least one immunosuppressive factor;

culturing the transformed or transfected host cells under conditions in which said cells express the recombinant polynucleotide molecule to produce said polypeptide wherein the polypeptide comprises at least a part of an immunomodulating agent; and

recovering said immunomodulating agent.

30. The method of claim 29 wherein said immunosuppressive factor is selected from the group consisting of a T cell receptor antagonist, a T cell receptor agonist and combinations thereof.

31. The method of claim 29 wherein said immunosuppressive factor is an analog of a peptide agonist capable of activating a T cell response to myelin basic protein.

32. The method of claim 29 wherein said Fc receptor ligand comprises at least a part of one domain of a constant region of an immunoglobulin molecule.

33. The method of claim 29 wherein said immunomodulating agent comprises a chimeric antibody.

34. The method of claim 33 wherein said chimeric antibody comprises a heavy chain wherein at least one complementarity determining region has been replaced with a T cell receptor antagonist.

35. A recombinant polynucleotide molecule encoding a polypeptide wherein said polynucleotide molecule comprises at least one nucleotide sequence corresponding to a Fc receptor ligand and at least one nucleotide sequence corresponding to an immunosuppressive factor.

36. The polynucleotide molecule of claim 35 wherein said immunosuppressive factor is selected from the group consisting of a T cell receptor antagonist, a T cell receptor agonist and combinations thereof.

37. The polynucleotide molecule of claim 35 wherein said polynucleotide molecule comprises a sequence corresponding to at least part of one domain of a constant region of an immunoglobulin molecule.

38. The polynucleotide molecule claim 37 wherein the immunoglobulin molecule is a human IgG molecule.

39. The polynucleotide molecule of claim 35 wherein said polynucleotide molecule encodes a nucleotide sequence corresponding to an immunoglobulin heavy chain wherein a complementarity determining region has been at least partially deleted and replaced with a nucleotide sequence corresponding to T cell receptor antagonist.

40. Transfected or transformed cells comprising a recombinant polynucleotide molecule according to any one of claims 35 to 39.

41. A method for the effective *in vitro* endocytic presentation of an immunosuppressive factor comprising the steps of:

providing a medium comprising a plurality of antigen presenting cells expressing Fc receptors; and

combining said medium with a immunomodulating agent containing composition wherein the composition comprises an immunomodulating agent having at least one Fc receptor ligand and at least one immunosuppressive factor and a compatible carrier.

42. The method of claim 41 wherein said Fc receptor ligand comprises at least part of one domain of a constant region of an immunoglobulin molecule.

43. The method of claim 41 wherein said immunomodulating agent comprises a polypeptide.

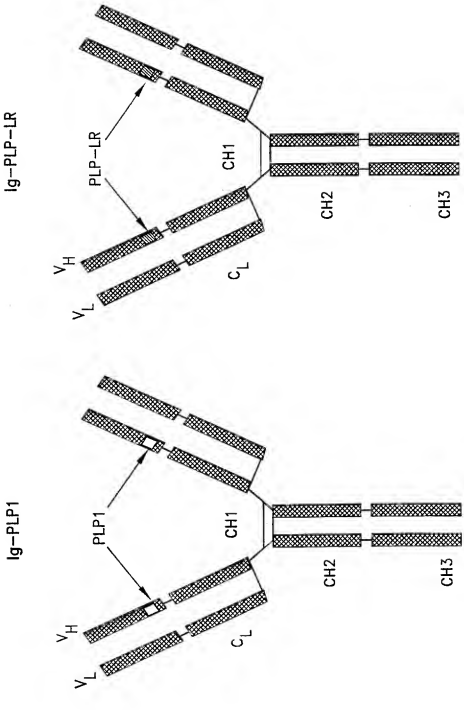

Ig-PLP1
PLP1
CH1
CH2
CH3
V_H
V_L
C_L
Ig-PLP-LR
PLP-LR
CH1
CH2
CH3
V_H
V_L
C_L

FIG. 1A

FIG. 1B

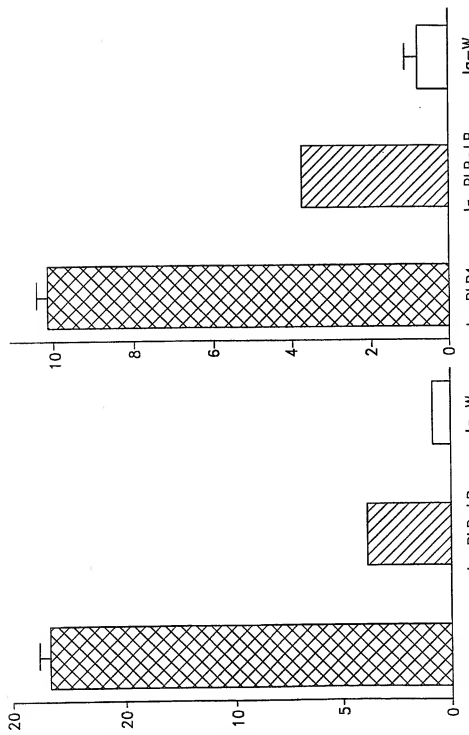

^{125}I–rat anti–mouse kappa bound (cpm x 10^{-3})
20
10
5
0
Ig–PLP1
Ig–PLP–LR
Ig–W
10
8
6
4
2
0
Ig–PLP1
Ig–PLP–LR
Ig–W

FIG. 2A

FIG. 2B

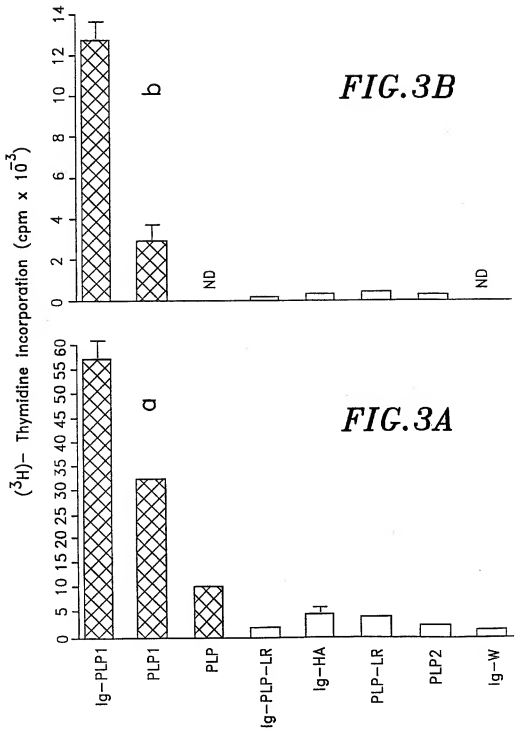

(^{3}H)- Thymidine incorporation (cpm x 10^{-3})
0 2 4 6 8 10 12 14
b
ND
ND
0 5 10 15 20 25 30 35 40 45 50 55 60
a
Ig-PLP1
PLP1
PLP
Ig-PLP-LR
Ig-HA
PLP-LR
PLP2
Ig-W

FIG.3B

FIG.3A

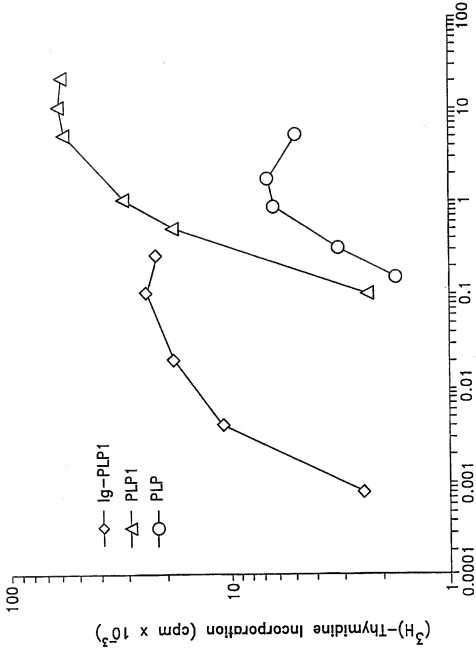

Ig-PLP1
PLP1
PLP
100
10
1
0.0001
0.001
0.01
0.1
1
10
100
Antigen (μM)
(³H)-Thymidine Incorporation (cpm x 10⁻³)

FIG. 4

FIG.5A

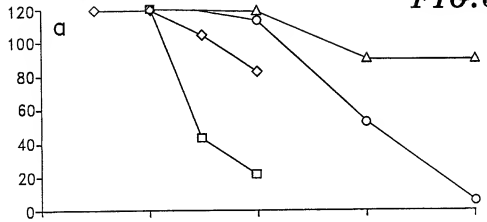


FIG.5B

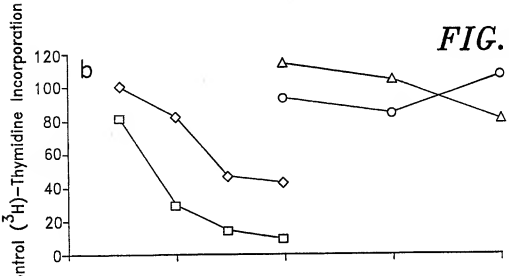
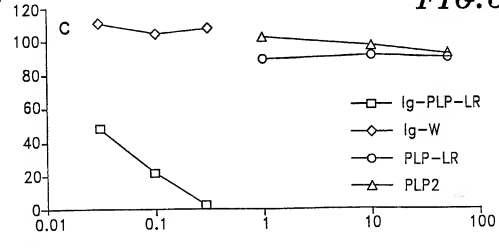


FIG.5C



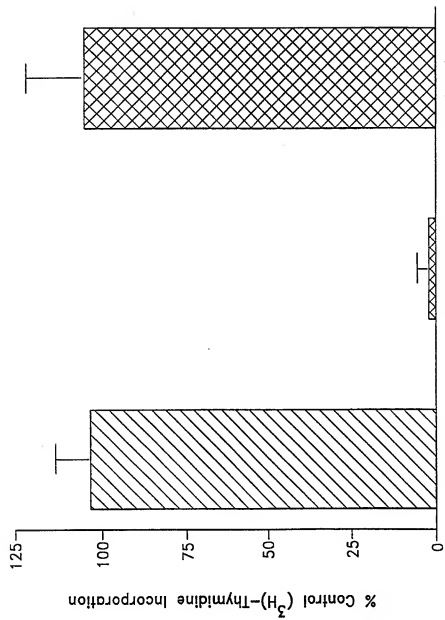

% Control (^{3}H)-Thymidine Incorporation
125
100
75
50
25
0
Ig-PLP2
Ig-PLP-LR
Ig-W

FIG. 6

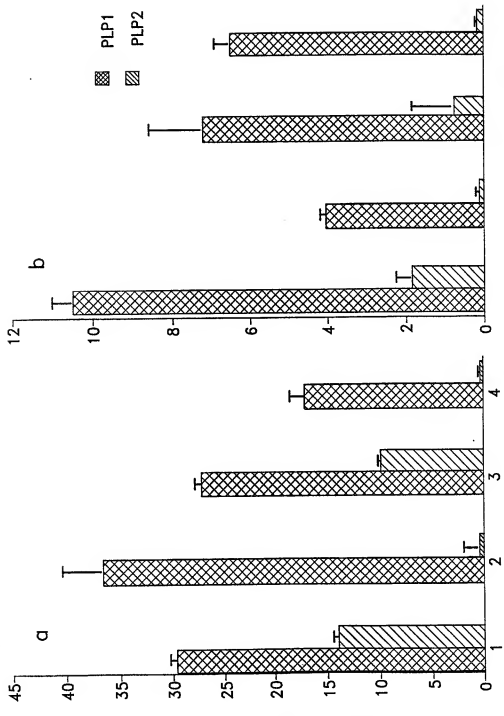

Thymidine incorporation (cpm x 10^3)
a
b
PLP1
PLP2
0
5
10
15
20
25
30
35
40
45
0
2
4
6
8
10
12
1
2
3
4

FIG. 7A

FIG. 7B

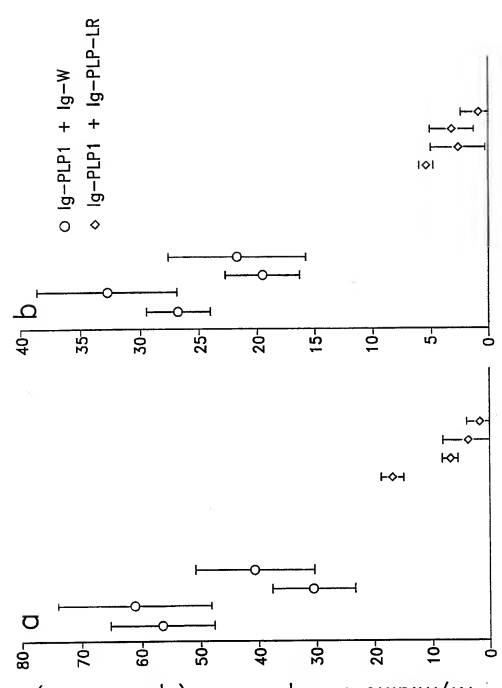

Thymidine incorporation (cpm x 10^{-3})
a
b
○ Ig-PLP1 + Ig-W
◇ Ig-PLP1 + Ig-PLP-LR
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30
35
40

FIG.8A

FIG.8B

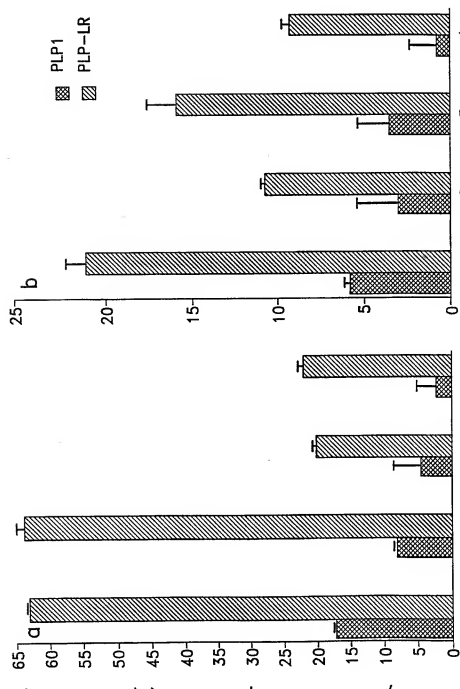

Thymidine incorporation (cpm x 10^3)
a
b
PLP1
PLP-LR
0
5
10
15
20
25
30
35
40
45
50
55
60
65
1
2
3
4

FIG.9A

FIG.9B

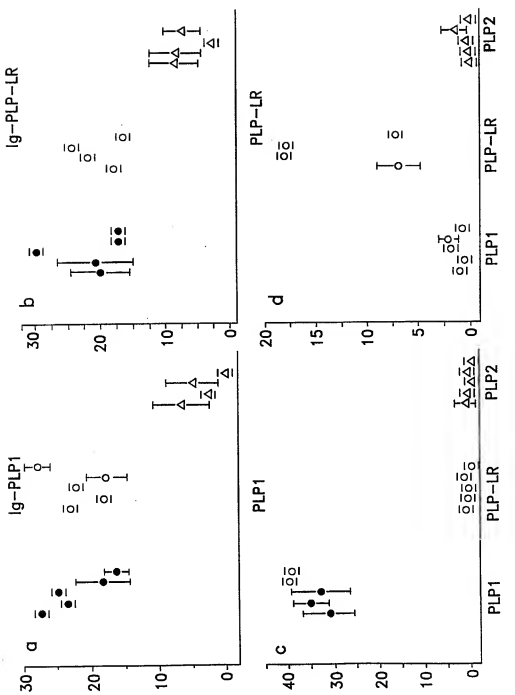

a
lg-PLP1
b
lg-PLP-LR
c
PLP1
d
PLP-LR
PLP1
PLP-LR
PLP2
PLP1
PLP-LR
PLP2
(3H)-Thymidine incorporation (cpm x 10-3)

FIG. 10

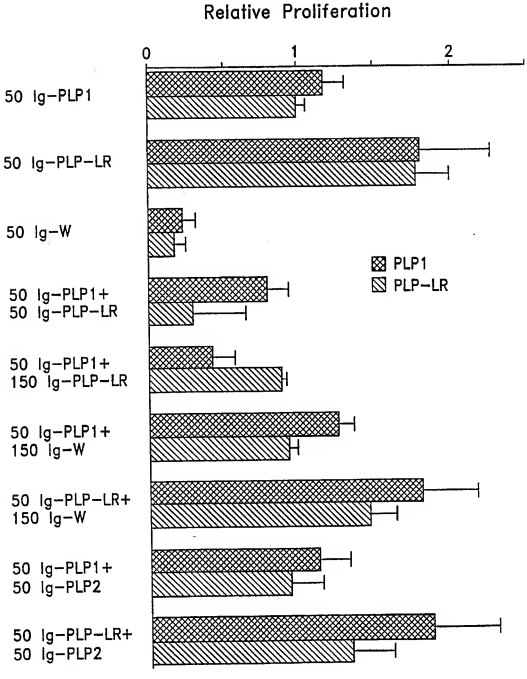

Relative Proliferation
0
1
2
50 Ig-PLP1
50 Ig-PLP-LR
50 Ig-W
50 Ig-PLP1+
50 Ig-PLP-LR
50 Ig-PLP1+
150 Ig-PLP-LR
50 Ig-PLP1+
150 Ig-W
50 Ig-PLP-LR+
150 Ig-W
50 Ig-PLP1+
50 Ig-PLP2
50 Ig-PLP-LR+
50 Ig-PLP2
PLP1
PLP-LR

FIG. 11

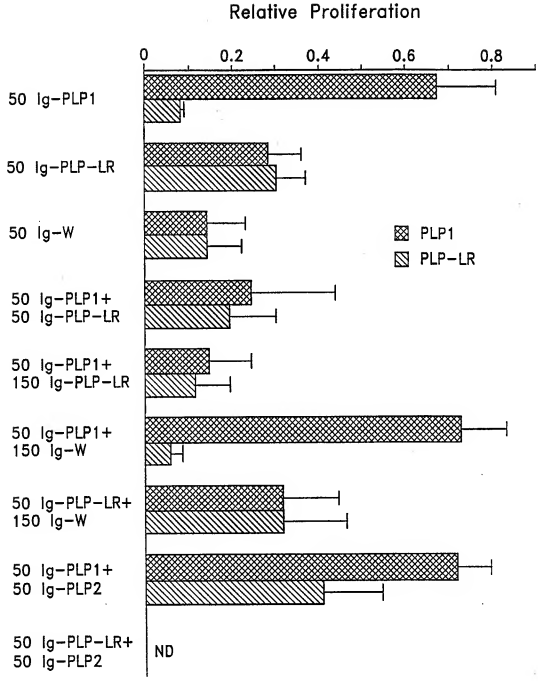

Relative Proliferation
0
0.2
0.4
0.6
0.8
50 Ig-PLP1
50 Ig-PLP-LR
50 Ig-W
50 Ig-PLP1+
50 Ig-PLP-LR
50 Ig-PLP1+
150 Ig-PLP-LR
50 Ig-PLP1+
150 Ig-W
50 Ig-PLP-LR+
150 Ig-W
50 Ig-PLP1+
50 Ig-PLP2
50 Ig-PLP-LR+
50 Ig-PLP2
ND
PLP1
PLP-LR

FIG. 12

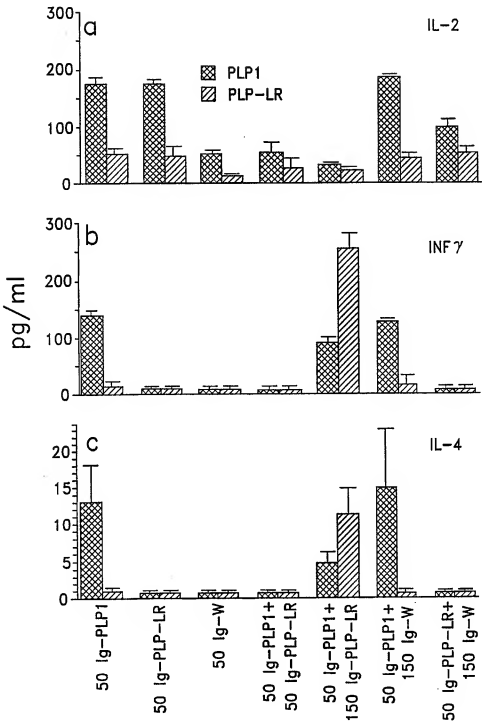

a
IL-2
PLP1
PLP-LR
300
200
100
0
b
INF γ
pg/ml
300
200
100
0
c
IL-4
20
15
10
5
0
50 Ig-PLP1
50 Ig-PLP-LR
50 Ig-W
50 Ig-PLP1+ 50 Ig-PLP-LR
50 Ig-PLP1+ 150 Ig-PLP-LR
50 Ig-PLP1+ 150 Ig-W
50 Ig-PLP-LR+ 150 Ig-W

FIG. 13

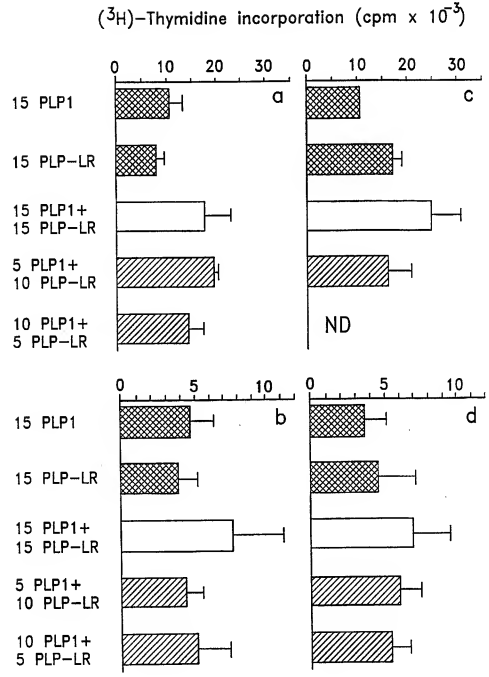

(^{3}H)–Thymidine incorporation (cpm x 10^{-3})
0 10 20 30
a
c
15 PLP1
15 PLP–LR
15 PLP1+
15 PLP–LR
5 PLP1+
10 PLP–LR
10 PLP1+
5 PLP–LR
ND
0 5 10
b
d
15 PLP1
15 PLP–LR
15 PLP1+
15 PLP–LR
5 PLP1+
10 PLP–LR
10 PLP1+
5 PLP–LR

FIG. 14

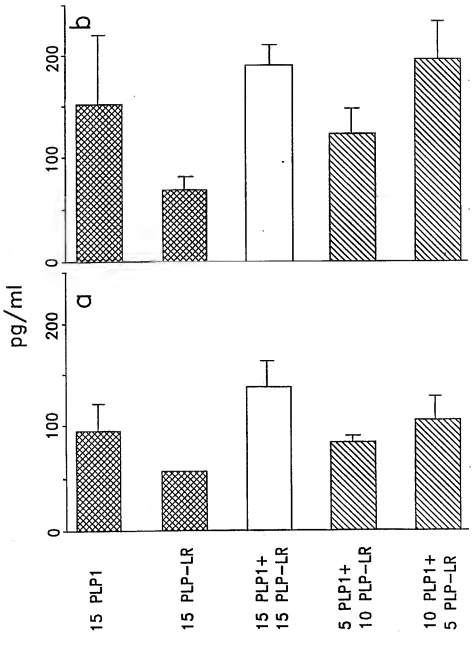

pg/ml
0
100
200
a
b
15 PLP1
15 PLP-LR
15 PLP1+
15 PLP-LR
5 PLP1+
10 PLP-LR
10 PLP1+
5 PLP-LR

FIG. 15

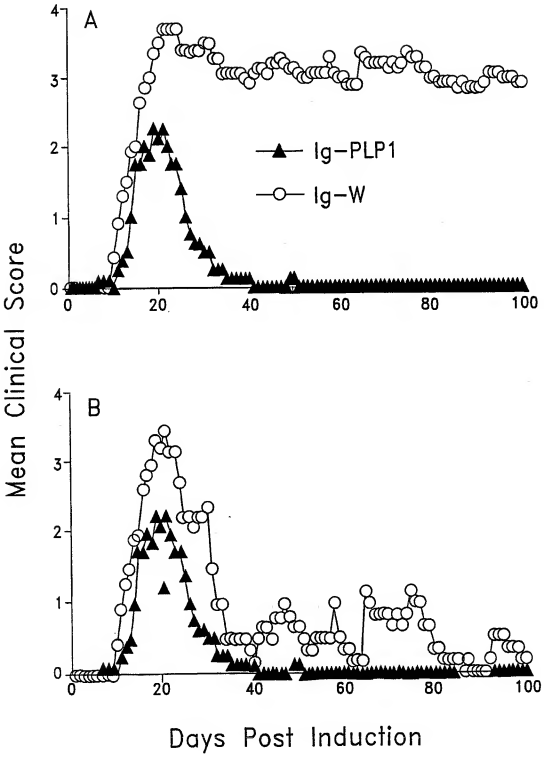

A
B
Ig-PLP1
Ig-W
Mean Clinical Score
Days Post Induction
0
1
2
3
4
0
20
40
60
80
100

FIG. 16

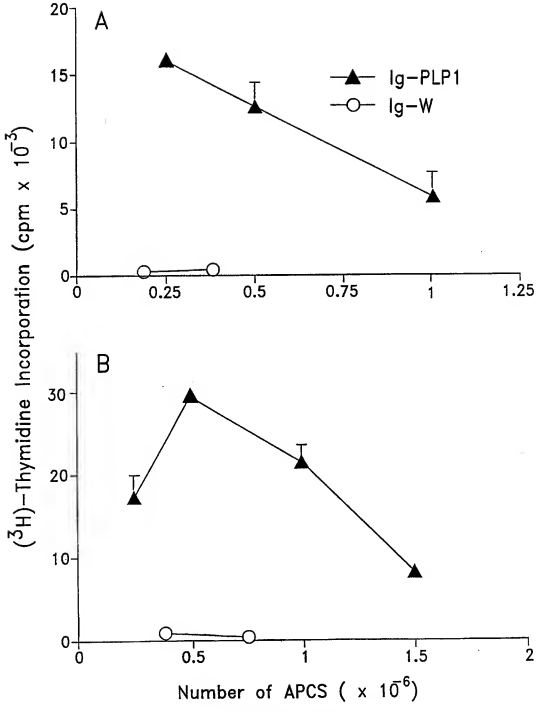

A
B
Ig-PLP1
Ig-W
(^3H)-Thymidine Incorporation (cpm x 10^{-3})
Number of APCS (x 10^{-6})
0
5
10
15
20
0.25
0.5
0.75
1
1.25
30
1.5
2

FIG. 17

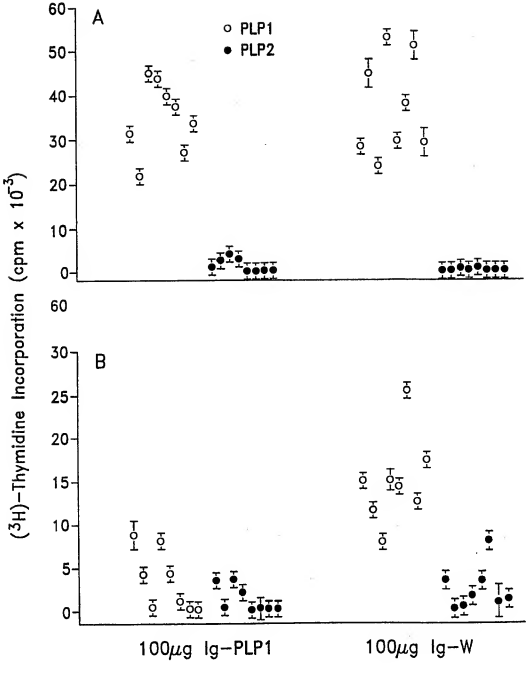

A
B
PLP1
PLP2
(3H)-Thymidine Incorporation (cpm x 10-3)
100μg Ig-PLP1
100μg Ig-W

FIG. 18

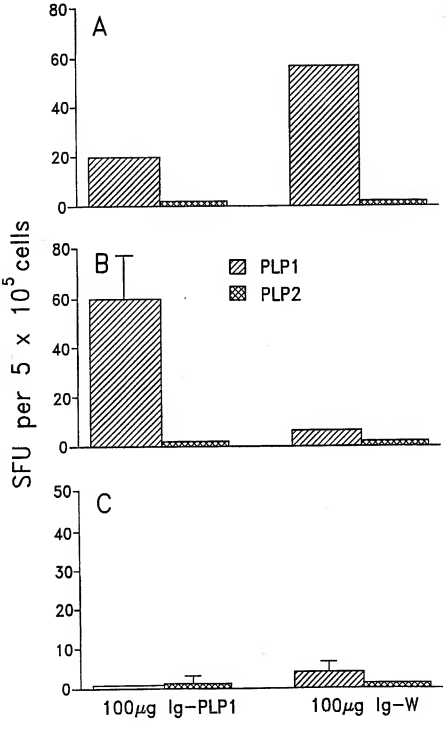

A
B
C
PLP1
PLP2
SFU per 5 x 10^5 cells
80
60
40
20
0
50
30
10
100μg Ig-PLP1
100μg Ig-W

FIG. 19

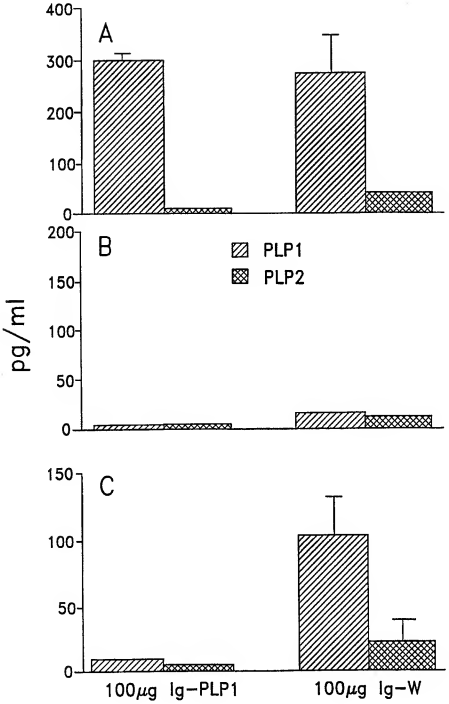

400
300
200
100
0
A
200
150
100
50
0
B
PLP1
PLP2
pg/ml
150
100
50
0
C
100μg Ig-PLP1
100μg Ig-W

FIG.20

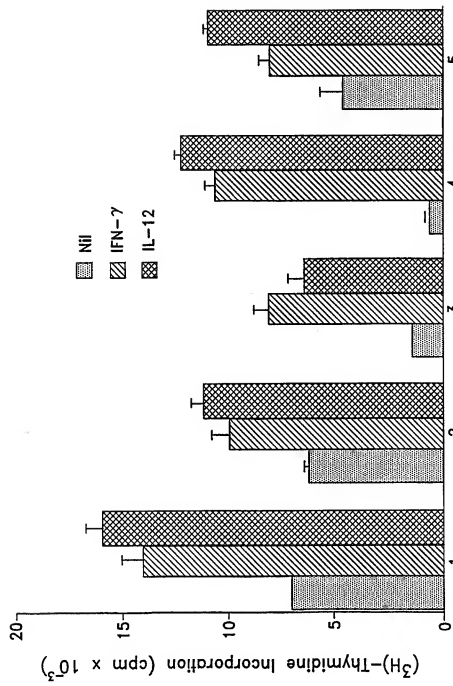

Nil
IFN-γ
IL-12
(^{3}H)-Thymidine Incorporation (cpm x 10^{-3})
0
5
10
15
20
1
2
3
4
5

FIG.21

INTERNATIONAL SEARCH REPORT

Inter "tional Application No
PCI/US 98/00520

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 A61K39/385 C07K19/00 C12N5/10

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MIN ET AL.: "Modulation in vivo of autoreactive T cells by a TCR antagonist Ig Chimera" JOURNAL OF THE ALLERGY AND CLINICAL IMMUNOLOGY, vol. 99, no. 1, January 1997, page S183 XP002065436 * see abstract No. 738 *	1-41
X	WO 94 28027 A (ARCH DEVELOPMENT CORP. USA) 8 December 1994 see the whole document -/--	1,11-13, 16,19, 23-29, 32,33, 35,38, 40-43

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 May 1998	08/06/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Muller-Thomalla, K

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INTERNATIONAL SEARCH REPORT

Inter: "onal Application No
PC1/US 98/00520

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 629 703 A (MEDAREX) 21 December 1994 see the whole document	1-41
A	WO 92 05793 A (MEDAREX) 16 April 1992 see the whole document	1-41
A	WO 94 10332 A (MEDAREX) 11 May 1994 see the whole document	1-41
A	WO 96 34622 A (ALEXION PHARM INC.) 7 November 1996 see the whole document	1-41
A	WO 93 06135 A (GENENTECH) 1 April 1993 see the whole document	1-41

1

INTERNATIONAL SEARCH REPORT

formation on patent family members

Inter onal Application No
PCT/US 98/00520

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9428027 A	08-12-94	AU 7098094 A	20-12-94
		CA 2163989 A	08-12-94
		EP 0700402 A	13-03-96
		JP 9501824 T	25-02-97
EP 0629703 A	21-12-94	US 4954617 A	04-09-90
		AT 120802 T	15-04-95
		AU 605771 B	24-01-91
		AU 7527187 A	14-01-88
		CA 1319899 A	06-07-93
		DE 3751214 D	11-05-95
		DE 3751214 T	16-11-95
		EP 0255249 A	03-02-88
		ES 2072851 T	01-08-95
		IL 101475 A	31-07-94
		JP 9316100 A	09-12-97
		JP 1500195 T	26-01-89
		JP 2648317 B	27-08-97
		WO 8800052 A	14-01-88
		US 5635600 A	03-06-97
WO 9205793 A	16-04-92	AU 667460 B	28-03-96
		AU 8869491 A	28-04-92
		CA 2093022 A	06-04-92
		EP 0553244 A	04-08-93
		JP 6502410 T	17-03-94
WO 9410332 A	11-05-94	AU 5542894 A	24-05-94
		CA 2148578 A	11-05-94
		EP 0668924 A	30-08-95
WO 9634622 A	07-11-96	AU 5565896 A	21-11-96
		EP 0830139 A	25-03-98
WO 9306135 A	01-04-93	EP 0605606 A	13-07-94
		JP 6511241 T	15-12-94

EXHIBIT E

Autoimmunity to Two Forms of Glutamate Decarboxylase in Insulin-dependent Diabetes Mellitus

Daniel L. Kaufman,* Mark G. Erlander,‡ Michael Clare-Salzler,§ Mark A. Atkinson,‖ Noel K. Maclaren,‖ and Allan J. Tobin¶**‡‡

*Department of Psychiatry and Behavioral Sciences; ‡Program for Neuroscience; §Department of Medicine; ‖Department of Biology; **Molecular Biology Institute; and ‡‡Brain Research Institute, University of California Los Angeles, Los Angeles, California 90024; and ¶Department of Pathology and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, Florida 32610

Abstract

Insulin-dependent diabetes mellitus (IDDM) is thought to result from the autoimmune destruction of the insulin-producing β cells of the pancreas. Years before IDDM symptoms appear, we can detect autoantibodies to one or both forms of glutamate decarboxylase (GAD_{65} and GAD_{67}), synthesized from their respective cDNAs in a bacterial expression system. Individual IDDM sera show distinctive profiles of epitope recognition, suggesting different humoral immune responses. Although the level of GAD autoantibodies generally decline after IDDM onset, patients with IDDM-associated neuropathies have high levels of antibodies to GAD, years after the appearance of clinical IDDM. We note a striking sequence similarity between the two GADs and Coxsackievirus, a virus that has been associated with IDDM both in humans and in experimental animals. This similarity suggests that molecular mimicry may play a role in the pathogenesis of IDDM. (*J. Clin. Invest.* 1992. 89:283–292.) Key words: insulin-dependent diabetes mellitus • glutamate decarboxylase • diabetic neuropathy

Introduction

Insulin-dependent diabetes (IDDM;[1] type I diabetes) is one of the most serious and common of metabolic disorders, affecting approximately 1 person in 300 in the U.S., while epidemiological studies in Europe suggest that its incidence is increasing (reviewed in 1–3). The disease is thought to result from the autoimmune destruction of the insulin-producing β cells of the pancreas and the subsequent metabolic derangements. Although insulin therapy allows most patients to lead active lives, this replacement is imperfect since it does not restore normal metabolic homeostasis. Metabolic abnormalities are thought to be important in the subsequent development of common complications, which include retinopathy, cataract formation, nephropathy, neuropathy, and heart disease.

While the initiating agent of IDDM autoimmunity is not known, it ultimately provokes a loss of immunological tolerance to self-antigens present in insulin-secreting β cells within the pancreatic islets (4–6). IDDM begins with an asymptomatic stage, characterized by a chronic inflammatory infiltrate of the islets (insulitis), which selectively destroys the β cells. Only after the destruction of the majority of the β cells, often occurring over several years, do hyperglycemia and ketosis appear.

The pathogenesis of IDDM involves both genetic and environmental factors. One or more susceptibility factors are encoded by the major histocompatibility complex on chromosome 6, probably by the DQ A1 and B1 loci (7, 8). Studies of monozygotic twins, however, show a concordance for IDDM of < 40%, suggesting that environmental factors play an important role (9). Long suspected environmental causes of IDDM include a number of viruses, such as rubella, encephalomyocarditis virus, and especially Coxsackie virus B_4 (reviewed in 10–12).

Autoantibodies to a 64,000 M_r islet cell protein are associated with IDDM and have been detected years before the onset of symptoms (13–15). Other IDDM-associated autoantibodies, such as those against insulin and cytoplasmic gangliosides of islet cells (ICA), appear later, possibly as a consequence of the release of these antigens (or their precursors) from the damaged islet cells (16, 17). Antibodies to the 64,000 M_r proteins are, however, the earliest and most reliable predictive marker of IDDM in humans and are also present in the two animal models for IDDM, the nonobese diabetic (NOD) mouse and the Biobreeding rat (14, 15, 18, 19).

Baekkeskov et al. (20) reported that the 64,000 M_r islet cell autoantigen is a form of glutamate decarboxylase (GAD; E.C. 4.1.1.15), the enzyme responsible for the synthesis of γ-aminobutyric acid (GABA) in brain, peripheral neurons, pancreas, and other organs (21). We have recently shown that the brain contains two forms of GAD, which are encoded by two separate genes (22). The two GADs (GAD_{65} and GAD_{67}) differ in molecular size (with M_rs = 65,000 and 67,000) and amino acid sequence (with ~ 30% sequence divergence), as well as in their intracellular distributions and interactions with the GAD cofactor pyridoxal phosphate (22–25). In brain neurons, GAD_{65} is preferentially associated with axon terminals, while GAD_{67} is present in both terminals and cell bodies (25).

Previous studies of the 64,000 M_r IDDM autoantigen have used pancreatic extracts enriched for membrane-associated

Dr. Erlander's present address is Department of Molecular Biology, Scripps Clinic, La Jolla, CA 92037. Address correspondence to Daniel L. Kaufman, Ph.D., Department of Psychiatry and Behavioral Sciences, UCLA, Los Angeles, CA 90024-1759, or to Allan J. Tobin, Ph.D., Department of Biology, UCLA, Los Angeles, CA 90024-1606.

Received for publication 22 March 1991 and in revised form 20 September 1991.

1. *Abbreviations used in this paper:* GAD, glutamate decarboxylase; ICA, islet cell antibodies; IDDM, insulin-dependent diabetes mellitus; JDF, Juvenile Diabetes Foundation; NIDDM, non-IDDM; NOD, nonobese diabetic; PAS, protein A-Sepharose; PCR, polymerase chain reaction.

J. Clin. Invest.

0021-9738/92/01/0283/10 $2.00
Volume 89, January 1992, 283–292

proteins. In view of our demonstration that the brain contains two GADs, we set out to determine the molecular identity of islet cell GAD by immunohistochemistry with monospecific antibodies. We then used GAD_{65} and GAD_{67} produced in genetically engineered bacteria from our GAD cDNAs to examine the specificity of IDDM autoantibodies for the two GADs and for restricted sets of GAD epitopes.

Our results lead to two new suggestions concerning the pathogenesis of IDDM and its complications: (*a*) GAD autoimmunity may play a role in the pathogenesis of IDDM-associated neuropathies; and (*b*) IDDM autoimmunity may result from molecular mimicry of GAD and a Coxsackievirus peptide.

Methods

Patient sera. IDDM patients and individuals at high risk for later developing IDDM were selected from a previous study at the University of Florida Diabetes Clinics (15, 26). IDDM patients with peripheral neuropathies were selected from the University of Florida Diabetes Clinics and the UCLA Diabetes Clinic.

Nondiabetic controls and the individuals studied before the documented clinical onset were ascertained through ongoing prospective screening for islet cell antibodies of more than 5,000 first-degree relatives of IDDM probands, and 8,200 individuals from the general population, of whom 4,813 were school children. These studies were approved by the University of Florida's Institutional Review Board. All participating individuals first gave their written informed consent. Individuals at high risk for the development of IDDM were identified by the presence of high titers of ICAs, assayed by indirect immunofluorescence on cryostat sections of blood group O human pancreas. All results were interpreted on coded samples, with control negative and positive sera in each batch. The ICA levels were estimated as Juvenile Diabetes Foundation units, according to the standardization guidelines established by the Immunology Diabetes Workshop (IDW), as previously described. M. Atkinson and N. Maclaren subscribe to the IDW's ICA proficiency testing program, which they currently supervise.

GAD assays. Patient sera were assayed blind for their ability to bind GAD enzymatic activity from a cleared homogenate of human cerebellar cortex in "GAD buffer," which contained 60 mM potassium phosphate, pH 7.1, 0.5% Triton X-100, 1 mM PMSF, 1 mM 2-aminoethylisothiouronium bromide, and 0.1 mM pyridoxal phosphate. IgG from each serum was bound to protein A-Sepharose (PAS) by adding 40 μl of serum to 80 μl of a 1:1 slurry of preswollen PAS in GAD buffer, incubating for 30 min at 4°C with gentle rocking, isolated by centrifugation, and then washing four times in the same buffer. 100 μl of brain extract was then added to each sample and incubated for 1 h at 4°C with gentle rocking, washed four times, resuspended in buffer, and assayed for GAD activity as previously described (25). Values shown are means of three determinations.

Immunohistochemistry. Immunochemical detection of the two forms of GAD was performed as previously described for rat cerebellum (25).

Antigen preparation and immunoadsorption. Rat GAD_{65} and GAD_{67} cDNAs were subcloned in the NcoI site of pET 8C and the NheI site of pET-5C respectively and transformed into *Escherichia coli* BL21 (DE3) (20, 27). Control and GAD-producing *E. coli* were grown and induced with isopropyl-thio-β-D-galactoside, harvested by centrifugation, resuspended in GAD buffer, sonicated, and cleared by centrifugation at 55,000 *g* for 15 min. For immunocompetition, 30 μl of each patient serum was incubated with 100 μl of extract from control bacteria or from bacteria that produced either GAD_{65} or GAD_{67} for 1 h at 4°C. Human pancreatic islets were labeled with ^{35}S-methionine as previously described (15). A detergent extract (300 μl) was first precleared with human control serum. The material that bound to the control IgG was removed with protein A-Sepharose. The precleared islet cell detergent extract was then split into three fractions and then incubated (2 h on ice) with serum that had been absorbed with each of the *E. coli* lysates. IgG-bound material was isolated with protein A-Sepharose as described above, and the bound material was analyzed by polyacrylamide gel electrophoresis in SDS (SDS-PAGE), followed by fluorography.

Detection of GAD autoantibodies. E. coli expressing rat GAD_{65} and GAD_{67} cDNAs were grown in minimal medium and induced with isopropyl-thio-β-D-galactoside in the presence of a mixture of ^{35}S-labeled amino acids (Tran-^{35}S; ICN Pharmaceuticals, Inc., Irvine, CA). The bacteria were harvested, sonicated in GAD buffer, and centrifuged to remove debris. Sera were preadsorbed with extracts of unlabeled host bacteria and then added to a mixture of ^{35}S-labeled extracts of GAD_{65}- and GAD_{67}-producing bacteria. IgG-bound polypeptides were isolated with PAS and analyzed by SDS-PAGE. Initial experiments analyzed sera for their ability to precipitate GAD_{65} and GAD_{67} separately (data not shown). Using a mixture of the two extracts simplified the assay. A number of *E. coli* polypeptides were also immunoadsorbed by some patient and some control sera. One such band, with M_r ~ 70,000, is apparent in many samples.

Epitope mapping. Portions of GAD_{65} cDNA were amplified by the polymerase chain reaction (PCR; 28) to produce DNA segments encoding three polypeptide segments: amino acid residues 1–224 (segment A); 224–398 (segment B); and 398–585 (segment C). Each construct also contained a T_7 promoter, a consensus sequence for the initiation of translation and an initiating methionine codon (29). Each PCR product was then transcribed in vitro with T_7 RNA polymerase and translated in vitro in a rabbit reticulocyte cell-free system in the presence of ^{35}S-methionine, using conditions recommended by the supplier (Amersham Corp., Arlington Heights, IL). Each test serum (30 μl) was incubated with the resulting ^{35}S-polypeptides. The bound peptides were isolated with PAS and analyzed by SDS-PAGE in 15% polyacrylamide and fluorography.

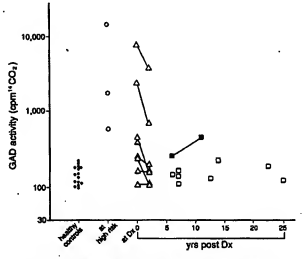

Figure 1. Immunoprecipitation of GAD activity by IDDM sera. GAD activity in brain extracts was immunoprecipitated with sera from healthy controls (●); individuals at high risk for IDDM (○); IDDM patients at diagnosis and two years later (△); and unrelated patients more than six years after diagnosis (□); one patient (■) developed a sensory neuropathy.

Results

IDDM patients have autoantibodies to GAD. We initially performed a blind trial to test for the presence of GAD autoantibodies in IDDM sera. We tested IDDM sera for the presence of GAD autoantibodies by assaying their ability to immunoprecipitate GAD activity from human brain homogenates (Fig. 1). We included sera from 35 individuals, which included 3 people judged to be at high risk for IDDM on the basis of their previously determined ICA titers, reduced responses to intravenous glucose, and their HLA DR/DQ haplotypes (15, 26), 8 IDDM patients studied at onset and 7 of these same patients two years later, 9 unrelated patients six or more years after IDDM onset, and 15 normal controls. Our results parallel those independently reported by Baekkeskov et al. (20).

The three high-risk individuals whose sera we examined had high anti-GAD titers, in one case comparable to those raised against purified brain GAD in experimental animals (data not shown). The levels of antibodies to GAD in five of eight newly diagnosed patients exceeded the mean + 1 SD of the control sera. Levels in these patients decreased by ~ 50% during the subsequent two years, with only two of seven sera having levels more than the mean + 1 SD of the control sera. In most patients ≥ 6 years after diagnosis, the concentrations of antibodies to GAD were indistinguishable from controls. In one patient in this series, however, anti-GAD levels actually rose between 6 and 11 years after onset, during which time the patient developed a sensory neuropathy.

Levels of anti-GAD antibodies in these patients generally parallel the previously determined titers of autoantibodies to the 64,000 M_r antigen. Our assays of immunoprecipitated GAD enzymatic activity easily identified individuals with high titers of autoantibodies to the 64,000 M_r antigen, but did not often distinguish individuals with low titers from controls.

This study established that autoantibodies to GAD are present at and before the clinical diagnosis of IDDM and decline within a few years after diagnosis. We next addressed the question of the molecular identity of the GAD autoantigen.

Islet cells contain both GAD_{65} and GAD_{67}. Immunohistochemical experiments with the GAD-6 monoclonal antibody, which recognizes only GAD_{65}, show the presence of GAD_{65} in pancreatic islets (Fig. 2; references 20, 25, 30). Using our recently described K-2 antiserum, which recognizes only GAD_{67}, we show that islet cells also contain GAD_{67} (Fig. 2; reference 25). Since both GAD_{65} and GAD_{67} are present in islets, either or both could be the autoantigen recognized by the IDDM sera surveyed in Fig. 1 and by Baekkeskov et al. (20).

The 64,000 M_r islet cell autoantigen is GAD_{65}. To define further the molecular identity of the IDDM autoantigen, we performed two sets of experiments. In the first experiment we used GAD-6 (the GAD_{65}-specific monoclonal antibody) to immunoadsorb GAD_{65} both from detergent extracts of ^{35}S-labeled islet cells and from soluble extracts of ^{35}S-labeled GAD-producing bacteria. GAD-6 specifically recognized a 65,000 M_r immunoreactive polypeptide in both islet cells and GAD_{65}-producing bacteria with identical electrophoretic mobilities, which were distinct from bacterially produced GAD_{67}. Prior immunoadsorption with an IDDM serum removes immunoreactive GAD_{65} (i.e., "64K") from both islet cell and bacterial extracts (data not shown).

In the second set of experiments, we examined the ability of bacterially produced GAD_{65} and GAD_{67} to compete with the immunoadsorption of islet cell autoantigens by IDDM sera. Sera taken from two patients (patient 052 and 496 which recognize both GADs; see Table I) specifically precipitate a polypeptide of M_r 64–65,000 from detergent-phase extracts of ^{35}S-labeled islets in the presence of extracts of host bacteria (i.e., bacteria not engineered to produce GAD), containing 400 μg of protein (Fig. 3, lanes *1* and *2*). When we added extracts (also containing 400 μg of total protein) of genetically engineered bacteria that produce either GAD_{65} or GAD_{67}, we found that an extract containing 100 μg of GAD_{67} partially blocked the binding of the islet cell antigen, as would be expected if GAD_{67} adsorbs some of the antibodies that recognize epitopes common to GAD_{65} and GAD_{67} (Fig. 3, lanes *5* and *6*). In contrast, an extract containing only 10 μg of GAD_{65} completely blocked immunoadsorption of the 64K autoantigen (Fig. 3, lanes *9* and *10*). These data show that the previously identified 64,000 M_r autoantigen is immunologically indistinguishable from GAD_{65}. A serum (patient 476) that predominantly recognizes GAD_{67} (which does not partition into the detergent phase of the islet cell extracts used in these studies) precipitated a very faint 64K band. The healthy control serum did not precipitate a 64K antigen.

IDDM sera differ in the recognition of GAD_{65} and GAD_{67}. Antisera raised in experimental animals against purified brain GAD vary in their recognition of GAD_{65} and GAD_{67}. With this in mind, we determined the specificity of individual IDDM sera for each species of GAD. We examined their ability to immunoprecipitate ^{35}S-labeled GAD_{65} and GAD_{67}, produced from GAD cDNAs in a bacterial expression system. We examined sera from 59 individuals (to whose IDDM status we were blind), including 8 people at high risk for IDDM, 12 people who later (3–64 months) developed IDDM, 3 newly diagnosed IDDM patients, 12 patients 2–22 years after onset who had no neurological symptoms, and 9 patients 10–48 years after onset who developed sensory or autonomic neuropathies (Table I; Fig. 4). None of the control sera from 15 healthy individuals had detectable ICA, antibodies to the 64,000 M_r pancreatic antigen, or antibodies to either form of GAD.

Levels of GAD autoantibodies were generally highest in the sera of individuals who were likely to have been in the process of developing the disease; those who were known to develop IDDM some time after their sera were drawn and those thought to be at high risk for IDDM on the basis of their previously determined ICA levels and autoantibodies to 64K. Levels were much lower in the sera of patients examined a few years after IDDM onset. None of the nine patients without neuropathies tested long after onset (≥ 5 years) had detectable antibodies to GAD. The intensity of the ^{35}S-labeled GAD_{65} immunoprecipitated by the IDDM sera generally paralleled the previously determined titers of autoantibodies to the 64,000 M_r islet cell polypeptide (15, 26), again supporting the latter's identification as GAD_{65}. There was no obvious correlation between ICA titers and the levels of autoantibodies to either GAD form.

Among IDDM sera, the ability to precipitate each of the two GADs varied among individuals. Of the 23 individuals tested whom we thought to be at early stages of IDDM (8 at high risk, 12 tested before subsequent onset, and 3 newly diagnosed), 15 recognized both GADs, 3 recognized only GAD_{65}, and 4 recognized only GAD_{67}. We found no obvious correlation between the time before or after diagnosis and the specific-

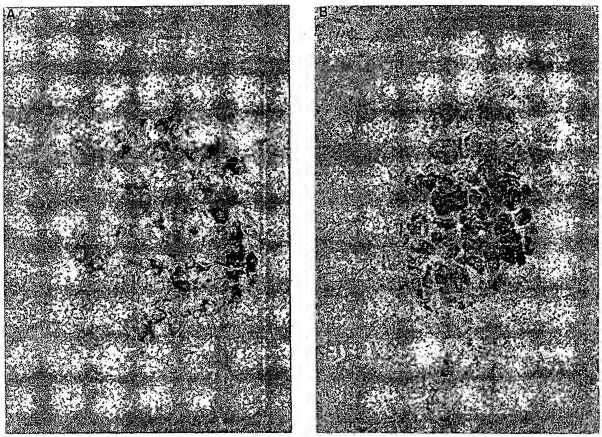

Figure 2. Pancreatic islets contain both GAD_{65} and GAD_{67}. (*A*) Immunohistochemistry with the GAD-6 monoclonal antibody specific for GAD_{65}, and (*B*) with the K-2 antiserum specific for GAD_{67}.

ity of autoantibodies for either form of GAD. A more sensitive assay (for example, one using human rather than rat GADs) might in fact reveal antibodies to both forms of GAD.

Altogether 78% (18/23) of sera from early stage IDDM individuals recognized GAD_{65}, a frequency similar to that reported in previous studies of 64,000 *M*, autoantibodies (reviewed in 15, 20). When we tested for both GADs, however, we could detect autoantibodies to either or both in 96% (22/23) of the early stage individuals tested.

The sera of NOD mice also show immunoreactivity both to GAD_{65} and to GAD_{67} (Fig. 4, lane *22*). This finding further underscores the similarity of the disease processes in human IDDM and in NOD mice.

Individual sera vary in epitope recognition. To examine the individual variability in epitope recognition of IDDM autoantibodies, we determined the ability of sera from four individuals to recognize three polypeptide segments of GAD_{65} (Fig. 5). Each of these individuals was at a different stage in the progression of the disease: 052 (high risk), 723 (a patient who subsequently developed IDDM), 705 (at diagnosis), and UC2 (advanced neuropathy). We used PCR amplification followed by in vitro transcription and translation of the PCR products to produce ^{35}S-labeled polypeptides that represented the amino-terminal (A), middle (B), and carboxy-terminal (C) thirds of GAD_{65}. None of the four sera reacted with the segment A, two (052 and UC2) reacted with segments B and C, one (705) with the carboxy-terminal segment C only, and one (723) with none of the GAD_{65} segments. Our inability to immunoprecipitate polypeptides with serum 723 (which, as shown in Fig. 4, does precipitate both GAD_{65} and GAD_{67} as intact molecules) may have resulted from a lack of sensitivity of the assay or from the inability of any of the utilized peptides to fold into the recognized epitope. While the three peptides that we investigated are unlikely to have formed all their native epitopes, our epitope mapping data, like our studies of the differential recognition of GAD_{65} and GAD_{67}, suggest that each of the tested sera has a distinctive profile of anti-GAD antibodies. Although IDDM autoantibodies recognize different GAD epitopes, we do not know which epitopes are recognized by the self-reactive T lymphocytes, which contribute to both humoral and cellular autoimmunity.

Persistent autoimmunity to GAD is often associated with peripheral and autonomic neuropathy. The occurrence of antibodies to GAD (and to the previously determined 64,000 *M*, antigen), is unusual in patients many years after onset (Fig. 1, Table I, and M. Atkinson, unpublished data). Autoantibodies to GAD were, however, present in 8 of 9 IDDM patients with sensory or autonomic neuropathies, long (10–41 years) after

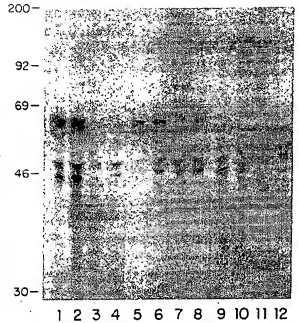

Figure 3. The 64,000 M_r autoantigen is GAD_{65}. The ability of sera which recognize both GADs (patients 052 and 496) to bind the previously described 64,000 M_r islet cell autoantigen was not blocked by preadsorption with an extract of wild-type BL21 (DE3) *E. coli*. Preadsorption with an extract of GAD_{65}-producing bacteria produced only partial blocking of these sera's ability to bind the pancreatic antigen. In contrast, preadsorption with extracts of GAD_{67}-producing bacteria, abolished the serum's ability to bind 64K antigen. Lanes *1–4* preadsorbed with 400 μg of a wild-type *E. coli* extract. Lanes *5–8* preadsorbed with a 400-μg extract containing 100 μg of GAD_{67}. Lanes *9–12* preadsorbed with a 400-μg extract containing 10 μg of GAD_{65}. Lanes *1, 5, 9*: patient 052. Lanes *2, 6, 10*: patient 496. Lanes *3, 7, 11*: healthy control. Lanes *4, 8, 12*: patient 476, whose serum predominantly recognizes GAD_{67} (Table I), does bind the 64K antigen very weakly which is not apparent in the photograph.

the onset of diabetic symptoms (Table I; Fig. 4, lanes *18–21*). Six of the sera examined had detectable levels of autoantibodies to both GAD_{65} and GAD_{67}, while two had detectable autoantibodies only to GAD_{67}. Two patients with rapidly progressing autonomic neuropathies (UC1 and UC2) had especially high levels of autoantibodies to GAD. In contrast, none of the nine patients who were free of IDDM-associated complications examined at or more than five years after onset had detectable antibodies to GAD. The GAD autoantibodies in neuropathy patients may result from the restimulation of the immune system by GAD released from damaged neurons, or they may be involved in the actual pathogenesis of this complication. In either case, GAD autoantibodies may serve as a useful marker of an ongoing degenerative process.

Sequence similarities between GAD and Coxsackievirus. Although we observe high levels of autoantibodies to GAD before IDDM onset, their presence may merely reflect an immune reaction to the exposure of previously sequestered antigens following β cell damage. Indeed, the initiating agent of the autoimmune response in IDDM is completely unknown, though the increasing incidence of IDDM and its frequent discordance in monozygotic twins has led to the suggestion that an environmental agent triggers autoimmunity (31, reviewed in 3, 12). In other autoimmune diseases, pathogenesis is thought to involve "molecular mimicry," in which a bacterial or viral antigen triggers an immune response that then reacts with a similar self antigen (reviewed 4, 32, 33).

Analysis of the deduced amino acid sequences of GAD_{65} and GAD_{67} shows an extensive and surprising sequence similarity to the P2-C protein of Coxsackievirus B_4. Coxsackievirus B_4 is a picornavirus with a worldwide distribution. It causes a mild upper respiratory infection and can also infect β cells (reviewed in 10–12). It has a small genome (7,395 bases), and its P2-C protein appears to contribute to the membrane-bound replication complex (34). A core polypeptide segment of six amino acid residues is identical in sequence between GAD_{65} and P2-C (Fig. 6; 22, 34). The immediately adjacent polypeptide segments also share a high level of similarity both in sequence and

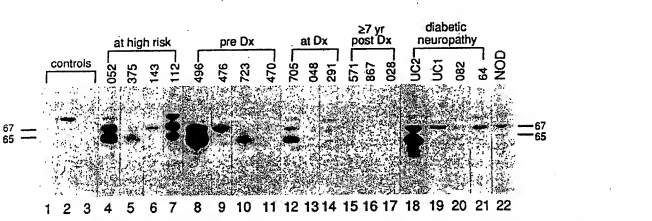

Figure 4. Detection of autoantibodies against GAD_{65} and GAD_{67} in IDDM sera. Sera were incubated with a mixture of ^{35}S-labeled lysates of GAD_{65}- and GAD_{67}-producing *E. coli*, and IgG-bound polypeptides were analyzed by SDS-PAGE. The composite photo shows representative data from controls and from individuals at different stages of IDDM: three controls (lanes *1–3*), four people at high risk for IDDM (lanes *4–7*), four who later developed IDDM (lanes *8–11*), three patients at diagnosis (lanes *12–14*), three IDDM patients more than seven years after diagnosis (lanes *15–17*), four IDDM patients with neuropathies (lanes *18–21*), and NOD mice (lane *22*).

Table I. Analysis of Characterized Sera for GAD_{65} and GAD_{67} Immunoreactivity

Patient ID				ICA	Anti-64K	Anti-GAD_{65}	Anti-GAD_{67}
				JDF units			
Individuals at high risk for IDDM							
052				160	+++	+++	+++
825				20	+++	++	0
375				0	+++	++	+
143				40	++	0	++
692				ND	++	0	+++
356				160	+++	++	+++
112				80	ND	+++	+++
410				0	++	++	++
Individuals who later developed IDDM							
	Months before IDDM diagnosis						
624	12			20	++	0	++
UF1	4			ND	+	+	++
584	24			0	++	+	0
035	64			40	+	+	0
496	6			160	+++	+++	+++
171	3			40	+	0	+
470	13			0	+++	+	+
055	8			40	+	0	0
438	42			320	++	+	+
840	9			0	+	+	+
723	14			ND	+++	+++	+
476	11			ND	+	+	+++
At onset of clinical symptoms							
048				320	+++	+	++
705				160	+++	+++	++
291				0	+++	++	++
IDDM patients without neuropathies							
	Years after diagnosis						
147	2			ND	+	0	0
476	3			0	ND	0	+
604	3			0	ND	+	0
113	5			160	ND	0	0
238	6			0	ND	0	0
997	6			80	ND	0	0
867	7			ND	ND	0	0
382	7			0	ND	0	0
052	12			0	ND	0	0
571	13			0	ND	0	0
M31	15			0	ND	0	0
025	22			0	ND	0	0
IDDM patients with neuropathies							
	Years after diagnosis	Autonomic neuropathy	Peripheral neuropathy				
UC1	35	+	+	0	ND	0	++
UC2	10	+	+	0	ND	+++	+++
UC3	13	+	+	0	ND	+	+
082	11	+	−	0	+	+	+

Table I. (Continued)

Patient ID				ICA	Anti-64K	Anti-GAD_{65}	Anti-GAD_{67}
038	21	+	+	ND	ND	+++	+++
344	33	−	+	0	ND	0	+
194	41	−	+	0	ND	+	+
310	48	−	+	0	ND	0	0
64	29	−	+	0	ND	+	++

Patient sera were obtained and assayed for ICA and autoantibodies to the 64,000 M_r protein as part of a previous study (15, 26) or from the UCLA Diabetes Clinic. Patients that are part of the University of Florida's database are identified by three digit numbers. Other patients are identified by sequential numbers, with UF numbers representing patients seen in Gainesville and UC patients seen in Los Angeles. ICA titers are expressed in JDF units. +++, high titers; ++, intermediate; +, detectable; ND, not determined. Patients UC1, UC2, and UC3 had rapidly progressing sympathetic neuropathies. None of the sera from 15 healthy controls had detectable ICA, antibodies to the 64,000 M_r protein, or antibodies to either form of GAD.

in the positions of charged residues. In the 24 residue segments of GAD_{65} and P2-C that are illustrated in Fig. 6, 19 residues are either identical or conservative differences. The three peptides shown in Fig. 6 have nearly identical hydrophobicity profiles (data not shown). The high charge density and the presence of a proline residue in the shared core suggest that the segments are highly antigenic. No other significant similarities were found between GAD and other viruses implicated in IDDM, such as rubella, mumps, encephalomyocarditis virus, and cytomegalovirus. A generally similar sequence similarity is also present in the P2-C region of other members of the Coxsackievirus family. If specific members of the Coxsackievirus family (such as B_4 and B_3) are indeed involved in the etiology of IDDM, their pathogenicity may involve factors such as their particular amino acid sequences, virulence, and cell trophism, as well as the host immune repertoire.

Discussion

In a blind clinical study, we tested IDDM sera for the presence of GAD autoantibodies by their ability to immunoprecipitate

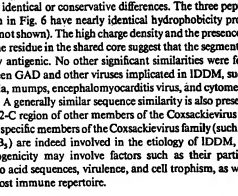

Figure 5. Epitope mapping of GAD_{65}. Three labeled segments containing the amino-terminal (*A*), middle (*B*), and carboxy-terminal (*C*) portions of GAD_{65} were immunoprecipitated with four IDDM sera that were initially characterized in the experiment shown in Fig. 4.

GAD enzymatic activity from brain homogenates. We found the highest levels of GAD autoantibodies in individuals at high risk for IDDM and in newly diagnosed IDDM patients. Levels of GAD autoantibodies decreased by ~ 50% within two years after diagnosis. Six years after IDDM onset, the patients whose sera we examined had GAD autoantibody levels indistinguishable from controls. One patient, however, displayed increased GAD antibodies years after onset, during which time the patient developed a sensory neuropathy.

Our studies of GADs in the brain have shown that neurons express two forms of GAD, which derive from separate genes (22). Pancreatic β cells also express GAD and use GABA to regulate glucagon secretion by α cells (35). Our immunohistochemical data, using antibodies monospecific for GAD_{65} and GAD_{67}, show that β cells, like most GABA neurons, contain both GAD_{65} and GAD_{67}. Although our enzymatic studies, and those of Baekkeskov et al. (20), demonstrated GAD autoimmunity in IDDM, they did not distinguish the two forms of GAD.

We used GAD_{65} and GAD_{67} cDNAs to express large amounts of each GAD in a bacterial expression system and tested the ability of each form to compete with the immunoadsorption of the 64,000 M_r autoantigen from ^{35}S-labeled islet cells. Only GAD_{65}-containing lysates effectively competed, suggesting that the 64,000 M_r autoantigen corresponds to GAD_{65}.

The islet cell homogenates previously used to characterize IDDM autoantigens were enriched for membrane-associated molecules and may preferentially have included GAD_{65}. In contrast, both our studies of the soluble fraction and those of Christie et al. (36) show a complex pattern of antigens recognized by IDDM autoantibodies (data not shown). Since islet cells contain both GAD_{65} and GAD_{67}, (Fig. 2) we sought to characterize the GAD autoantibodies by testing the ability of IDDM sera to recognize bacterially produced GAD_{65} and GAD_{67}.

We could detect autoantibodies to either GAD_{65} or GAD_{67} or both in almost all people who later developed IDDM, in some cases years before the onset of clinical symptoms. Of 23 early stage IDDM individuals tested, we found antibodies to both GADs in 15, to GAD_{65} alone in 3, and to GAD_{67} alone in 4. By testing for antibodies to both forms of GAD we were able to detect GAD antibodies in 96% of the individuals tested.

Figure 6. GAD and Coxsackievirus P2-C share common sequences. Solid line encloses identical amino acid residues. Dashed line encloses amino acid residues with similar charge, polarity, or hydrophobicity. Numbers refer to the amino acid residues in GAD_{65}, GAD_{67}, and Coxsackievirus protein P2-C. The human GAD amino acid sequences, which are almost identical to the rat GAD sequences, were determined by Bu Dingfang et al. (manuscript submitted for publication).

Levels of GAD autoantibodies were usually highest before IDDM onset and, in our patient sample, appeared as early as five years before onset of symptoms. GAD antibody levels declined after onset, presumably in parallel with the loss of GAD-containing β cells and the extinction of antigen-driven autoimmunity.

Patients showed varying immunoreactivity to GAD_{65} and GAD_{67}, which share ~ 70% amino acid similarity and are most divergent at their amino termini (22). They also varied in their ability to recognize individual polypeptide segments of GAD_{65}. These data suggest a diverse B lymphocyte response to different epitopes of GAD. While not all IDDM sera recognize the GAD_{65} polypeptide that contains the sequence shared with Coxsackievirus (segment B in Fig. 5), the antibodies may recognize GAD epitopes different from those that originally activated T lymphocytes.

Because our initial survey of IDDM patients detected increased levels of GAD autoantibodies in a patient who developed a sensory neuropathy long after the onset of diabetes itself, we further studied GAD autoimmunity in patients with IDDM-associated neuropathies. We found that 8/9 patients who had developed clinical IDDM symptoms 10–41 years earlier, showed significant levels of autoantibodies to GAD_{65}, GAD_{67}, or both. Of the eight patients in this group for whom we had ICA data, none had detectable ICA, and their low basal C-peptide did not respond to intravenous glucagon, suggesting that the continued high levels of anti-GAD autoantibodies did not result from the persistence of GAD-containing β cells.

The production of anti-GAD autoantibodies in patients with diabetic neuropathy may reflect continued stimulation of the immune system by GAD in the peripheral nervous system. Consistent with this hypothesis, Rabinowe et al. (37) have shown that, in some IDDM patients, autoantibodies to sympathetic ganglia are present at the diagnosis of IDDM, before the onset of clinical neuropathy. In addition, postmortem examination has revealed lymphocytic infiltration of sympathetic ganglia in IDDM patients with autonomic neuropathy (38). Our data suggest that autoimmunity to GAD, together with the metabolic effects of hyperglycemia, may play an important pathogenic role in diabetic neuropathy in IDDM.

The surprising similarity of the amino acid sequences of GAD_{65} and GAD_{67} to the P2-C protein of Coxsackievirus suggests that IDDM autoimmunity may arise by molecular mimicry, as a consequence of infection by Coxsackievirus. Epidemiological studies have shown that 39% of newly diagnosed IDDM patients have IgM responses to Coxsackievirus, compared to 6% of controls (39, 40). The molecular mimicry hypothesis suggests a mechanism to explain both the epidemiological association of Coxsackievirus B_4 with human IDDM and its ability (in contrast to other viruses epidemiologically associated with IDDM) to produce diabetes in mice and primates (31, 41–44). Direct association of Coxsackie B_4 infection and subsequent onset of human IDDM has been documented in a few cases (45, 46).

Coxsackievirus infection (perhaps of β cells themselves) may, in genetically susceptible individuals, initiate the characteristic autoimmune attack on pancreatic β cells. Viral peptides would then be presented to T lymphocytes, probably on the surface of antigen-presenting cells in the context of class II molecules. Although the sequences of both GADs suggest that they are cytosolic molecules, GAD polypeptides may be presented on the cell surface in the context of MHC molecules (as discussed in 47 and 48). Christie et al. (36), moreover, have demonstrated the association of the molecule we now know to be GAD_{65} with β cell membranes. GAD epitopes on the surface of β cells, in the context of either class I or class II molecules, could thus become the targets of immune responses initially directed against a Coxsackievirus epitope. The resultant destruction of β cells would then release more GAD_{65} and GAD_{67}, including GADs from the cytoplasm. The released GAD could then continue to stimulate lymphocytes already primed to the Coxsackievirus peptide, thus perpetuating the immune response long after the termination of the viral infection. This molecular mimicry would then lead to the continued autoimmune destruction of β cells and eventually to the development of clinical diabetes.

Assays for antibodies to recombinant GADs should allow a straightforward means of distinguishing IDDM from other forms of diabetes mellitus. This should be especially useful for evaluating adult patients presenting with the more common type II, non-insulin-dependent diabetes mellitus (NIDDM). Patients with true NIDDM do not have ICAs or autoantibodies to the 64,000 M_r protein or to insulin. Of adult onset patients initially diagnosed as having NIDDM, however, 10–15% are true type I (IDDM) diabetics and will eventually require insulin therapy.

Clinical trials are now under way to test the effectiveness of general immunosuppressive agents (such as cyclosporin and azathioprine) in delaying the onset of IDDM in individuals at high risk, that is, who already have islet cell autoantibodies

(49). Autoantibodies to GAD are the earliest indication of autoimmunity in IDDM and the two GADs are therefore excellent candidates for the initial targets for autoimmunity. Future experiments will determine whether the epitope shared by GAD and Coxsackievirus contributes to IDDM pathogenesis. If GAD is indeed involved in the etiology of IDDM, it may be possible to devise specific, rather than nonspecific, immunosuppressive strategies to block the function of specific MHC and T cell receptor molecules.

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EXHIBIT F

AN 1999:597309 PROMT
TI AutoImmune shares collapse on Colloral data in rheumatoid arthritis.
SO Marketletter, (13 Sep 1999) .
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PB Marketletter Publications Ltd.
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TX US biotechnology firm AutoImmune saw its shares crash 74% on September 1 to close at $1.40 following the announcement that its oral tolerance drug Colloral (collagen) had failed in Phase III development. By the end of the trading week (September 3), the firm's stock was selling at a miserly $0.84, a nasty turnaround for a company which was riding high on the back of its oral tolerance technology a couple of years ago with stock being traded around the $14 mark.

AutoImmune says that Colloral will be dropped from development and the firm will "immediately reduce its headcount and other operating expenses to conserve resources as we evaluate our strategic options to maximize shareholder value." The company told the Marketletter that it plans to cut its workforce by 96%, downsizing to eight staff from 26 immediately and then to two employees by the end of the month.

In the 772-patient trial, Colloral was found to be safe but did not meet the primary endpoint, which the spokeswoman said was achieving statistical significance in three out of the "core-four" parameters (tender joints, swollen joints, physician's global assessment and patient global assessment). While AutoImmune says that "substantial improvements" from baseline were observed in each of these measurements, the placebo response was "much greater than previously observed." In fact, the spokeswoman noted that, although the data were not publicly available at present, the placebo response was two times higher than in previous studies of the drug. Full data may be presented at a forthcoming rheumatology meeting, and the firm is considering switching the focus of Colloral to a nutraceutical product.

When asked whether the trial could be designed differently, the spokeswoman told the Marketletter that it "was perfect." Financially, however, the firm cannot keep funding the clinical development of Colloral. AutoImmune had continued its clinical development of the drug even though earlier trials had failed to demonstrate strong data. Two years ago, the company revealed that two Phase II trials of Colloral in RA had failed to yield statistically significant results (Marketletter May 19, 1997). However, the firm decided to pursue Phase III development following an independent re-analysis by statisticians who concluded that the drug was significantly more effective than placebo (Marketletter September 15, 1997).

General expectations for the drug were not high, particularly following the earlier failure of another mucosal tolerance program, Myleral (myelin basic protein) for multiple sclerosis which performed no better than placebo in Phase III trials.

Yet some investors may see this a good buying opportunity, with analysts pointing out that the company has a decent cash position with few liabilities; as of June 30, the firm had cash and cash equivalents of almost $9.7 million and the spokeswoman added that once liabilities have been paid, this will be down to around $7 million.

Ideal opportunity to buy?

Furthermore, AutoImmune has a very strong intellectual property position and is still conducting a number of other trials which are funded

externally. These include studies in new-onset type 1 diabetes (with Eli Lilly) and a pilot trial in chronic organ transplant rejection (results from both are due next year). Enrollment is continuing in a National Institutes of Health-funded long-term prevention study for type 1 diabetes.

Importantly, the firm also has an exclusive agreement with Teva Pharmaceutical for applications of AutoImmune's proprietary technology. The deal covers the development of an oral formulation of Teva's injectable multiple sclerosis drug Copaxone (glatiramer acetate) and an oral product for the treatment of myasthenia gravis, for which AutoImmune will receive milestone payments on product approval and royalties on any future sales. Teva is getting ready to start a Phase II/III trial of oral Copaxone with the first patient expected to be enrolled by year end, while the product for myasthenia gravis is also due to begin clinical development before the end of the year.

Despite speculation that the company's faith in the potential of inducing oral tolerance to antigens as a means of treating autoimmune disease may be misguided, AutoImmune says it still firmly believes in its technology. In a statement, the company said that "both basic and clinical research focused on enhancing the biological effect of (mucosal tolerance therapy) in patients will continue."

AutoImmune is currently assessing a number of different plans, including possible mergers and converting into a shell company while waiting for clinical data from its other ongoing trials to come through. The spokeswoman said that there has been interest from some firms in a merger, particularly as AutoImmune has such a strong IP position.

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EXHIBIT G

Immunology 2001 104 367–376

REVIEW ARTICLE

Peptide-based immunotherapy of autoimmunity: a path of puzzles, paradoxes and possibilities

STEPHEN M. ANDERTON *Institute of Cell, Animal and Population Biology, University of Edinburgh, Ashworth Laboratories, Edinburgh, UK*

INTRODUCTION

Our understanding of the basic mechanisms controlling the generation of productive immunity continues to evolve at a great pace. Models of how the immune response may be diverted from tackling invading pathogens into mounting an inappropriate response towards self tissue antigens also continue to develop. Armed with this knowledge, however, we are yet to develop effective and broadly applicable strategies to prevent or treat autoimmune disorders. This review assesses the prospects for developing antigen-specific therapies through the use of synthetic peptide antigens, and specifically altered peptide ligands (APL), to target pathogenic T-cell autoreactivity.

Identification of T-cell epitopes within autoantigens has allowed the development of a minimalist approach to certain experimental models. Thus single peptides can replace purified autoantigen or homogenized tissue as the agents that elicit T-cell autoreactivity and therefore pathology. If we are using peptides to provoke disease, we should also be able to use peptides to prevent disease, and many reports over the last 15 years have confirmed this. But is this applicable to human autoimmune disorders and what is the best approach to use?

ALTERED PEPTIDE LIGANDS: DEFINITIONS AND ACTIVITIES

By using analogue peptides with defined substitutions at individual residues, we can determine residues that interact either with the T-cell receptor (TCR) (analogues do not stimulate antigen-specific T cells but retain the ability to bind major histocompatibility complex (MHC)) or with the MHC (loss of both T-cell stimulation and MHC binding). The term 'altered peptide ligand' was first coined a decade ago by Evavold *et al.* to define peptides with alterations at TCR binding residues.[1] The definition of APL has since become broadened to include analogue peptides containing one or more substitution(s) at any residue. APL can be divided based on their ability to stimulate antigen-specific T cells (Fig. 1). The native peptide or APL that give equivalent response patterns are referred to as agonists. APL requiring increased doses to induce qualitatively normal responses are weak or subagonists, whilst those able to stimulate at greatly reduced doses are superagonists. The original work by Allen and colleagues identified APL with 'partial agonist' activity, being able to stimulate effector function (cytokine production, target cell lysis) in T-cell clones in the absence of the concomitant proliferation provoked by agonist peptides.[2,3] Soon after, Sette's group reported APL with alterations at TCR contact residues that did not overtly stimulate T-cell clones, but inhibited activation induced by agonist when presented on the same antigen-presenting cell (APC).[4,5] This inhibition was not simply due to MHC blockade and was antigen-specific (i.e. an APL of antigen *x* would not inhibit activation of T cells specific for antigen *y*). APL with these inhibitory properties are termed TCR antagonists. APL have proved useful tools in the dissection of early signalling events proximal to TCR ligation. Debate continues over the precise mechanisms underlying partial agonist and TCR antagonist effects and is documented elsewhere.[6,7]

APL AND IMMUNOTHERAPY: EARLY EXPERIMENTS

Pioneering work in the field of peptide-induced modulation of autoimmunity used murine experimental autoimmune encephalomyelitis (EAE)[8] which serves as a model for multiple sclerosis (MS). EAE is induced by immunization with complete Freund's adjuvant (CFA) mixed with antigens of central nervous system (CNS) myelin, notably myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). Central to the disease is activation of myelin-reactive $CD4^+$ T helper type 1 (Th1) cells which infiltrate the CNS and establish an inflammatory lesion. As there are several well-defined T-cell epitopes in these myelin antigens[9–12], the disease can be induced using single peptides. For this reason it is the EAE model that has seen the most extensive investigations of peptide therapy. Three immunodominant regions have been of particular interest: PLP(139–151) and two regions within MBP, the acetylated N-terminal peptide (Ac1–9) and peptides contained within 80–105.

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Abbreviations: APL, altered peptide ligand; CFA, complete Freund's adjuvant; Copl, co-polymer 1; EAE, experimental autoimmune encephalomyelitis; IFA, incomplete Freund's adjuvant; MBP, myelin basic protein; MG, myasthenia gravis; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PLP, proteolipid protein; TCR, T-cell receptor.

Correspondence: Dr S. M. Anderton, Institute of Cell, Animal and Population Biology, University of Edinburgh, Ashworth Laboratories, King's Buildings, West Mains Road, Edinburgh, EH9 3JT, UK. E-mail: steve.anderton@ed.ac.uk

The first epitope to be identified,[9] and consequently tested as a therapy,[13] was MBP Ac1–9. The molecular basis for the interaction of Ac1–9 with the A^u MHC class II molecule and TCR has been characterized extensively[13–15] and is summarized in Fig. 2. The residues responsible for binding to A^u are 4Lys and 5Arg.[13] However, the natural peptide binds extremely poorly,[9,16,17] due to 4Lys interacting unfavourably with a

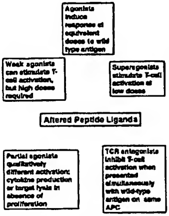
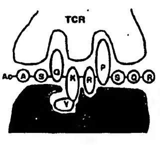


Figure 1. Altered peptide ligands: subtypes and activities. APL can be divided according to their effects on antigen-specific T-cell populations *in vitro*.



Peptide	Relative binding Affinity for A^u	Activation threshold for Ac1–9-specific T cells
4Lys (wild-type)	1	10 nm
4Ala	1000	10 pm
4Val	20 000	100 fm
4Tyr	100 000	1 fm

Figure 2. How the Ac1–9 peptide interacts with TCR and MHC class II. The Ac1–9 peptide of MBP binds to the A^u class II molecule through interactions involving residues 4Lys and 5Arg. Residue 4 has a dominant effect on binding, with the wild-type Lys residue interacting unfavourably with the A^u peptide-binding cleft. APL with alterations at position 4 can greatly increase binding to A^u. These APL consequently act as superagonists *in vitro*, stimulating T cells raised against wild-type Ac1-9 at correspondingly low concentrations. Residues 3 and 6 interact with TCR. This scheme represents interactions with the Tg4 TCR for which residues 6 and 3 act as primary and secondary TCR contact residues, respectively. This preference in TCR contacts is reversed on the analysis of polyclonal Ac1–9-specific populations. This figure summarizes data from refs 13–15 and 20.

hydrophobic pocket within the A^u peptide binding cleft, as highlighted by mutational studies and predictive computational analysis.[18,19] Hence we can produce APL with greatly increased binding affinities for A^u by substitution of residue 4Lys, most notably with Ala, Val and Tyr[13,15,16] (Fig. 2). As we would predict, increased binding for class II translates into increased capacity to stimulate Ac1–9-specific T cells *in vitro*,[13] such that femtomolar doses of the 4Tyr APL will stimulate responses that require nanomolar concentrations of wild-type Ac1–9.[20] Thus these position 4 APL behave as superagonists.

The initial peptide therapy experiments of Wraith *et al.* focused on the 4Ala APL and uncovered a conundrum.[13] Whilst the Ac1–9 4Ala peptide was a superagonist *in vitro*, it induced little or no EAE *in vivo*. Moreover, mice that were co-immunized with a mixture of 4Ala and wild-type 4Lys also showed little EAE. Preimmunization with 4Ala in incomplete Freund's adjuvant (IFA) also prevented EAE development on immunization with 4Lys in CFA.[21] This was not due to the expansion of a regulatory population as T cells from 4Ala-primed mice could not transfer protection from EAE to syngeneic recipients. The basis for these paradoxical *in vitro* and *in vivo* effects has remained unclear for a decade but our recent data explain these findings (see below). Was this phenomenon peculiar to the Ac1–9 model? Later studies from van Eden's laboratory using EAE and adjuvant arthritis in rats suggested that it may be generally applicable.[22] APL of MBP(72–85) and the arthritis-related peptide 180–188 of mycobacterial heat-shock protein 65 (hsp 65) were generated that showed increased binding affinities for the RT1B[l] rat class II molecule. In co-immunization experiments it was found that the MBP APL specifically inhibited EAE but not arthritis, indicating direct effects on antigen-specific T cells. These early experiments therefore pointed to applications for APL in antigen-specific therapy of autoimmune disorders.

TCR ANTAGONISM AND AUTOIMMUNITY

The idea of TCR antagonist peptides as therapeutics was first applied to the EAE model induced with the immunodominant PLP[139–151] epitope in SJL mice.[23] Residue 144Trp was identified as the dominant TCR contact for this epitope. Position 144 APL were identified that inhibited the *in vitro* activation of encephalitogenic 139–151-specific T-cell clones. When pools of these antagonist APL were added in equimolar amounts to the wild-type 139–151 prior to immunization in CFA, they were found to reduce significantly the incidence and severity of resulting EAE. Subsequently the approach was modified to design a single APL with substitutions at both 144 and 147.[24] This L144/R147 APL inhibited *in vitro* activation of a panel of encephalitogenic T-cell clones showing distinct TCR gene usage. The analogue prevented EAE when co-administered with native 139–151 and, furthermore, could limit progression of EAE if given early after the onset of disease.

Human autoreactive T cells can also be modulated by APL. T-cell reactivity to a mitochondrial 38 000 MW islet antigen has been described early after onset of type I diabetes.[25] APL based on this antigen were found to act as TCR antagonists when presented with wild-type antigen.[26] T cells derived from myasthenia gravis (MG) patients were found to respond to two epitopes within the human acetylcholine receptor (AChR) α subunit. APL of these two peptides, or a hybrid APL

combining both analogues in a single peptide, showed effective antagonist activity on MG T-cell responses to wild-type AchR peptides.[29] These findings were reproduced in an experimental model of MG in which APL prevented the development of clinical signs.[28,30] Several studies have also reported APL-induced modulation of T cells derived from MS patients and specific for region 80–100 of MBP.[31–33]

There is a conceptual problem, however, with the use of TCR antagonist APL as therapeutic tools. Whilst antagonists are clearly capable of inducing some early signalling events, these do not appear to have long-lasting profound effects on T-cell reactivity (although partial agonist APL may anergize T-cell clones *in vitro*[34]). Antagonist peptides inhibit when presented on the same APC as the agonist peptide (and usually when presented in molar excess). How then are we to achieve effective treatment with antagonists? They should have no effect on potentially autoreactive T cells in the absence of the native self antigen and therefore would not be a good option for prophylaxis. As treatments for active disease it is not inconceivable that antagonist and self antigen could be presented on the same APC. This would most likely happen in peripheral lymphoid organs however, and not in the affected organ where pathogenic self-reactive T cells would be active. Perhaps a more efficacious approach therefore is to design APL that convert autoreactive T cells to a benign or protective functional phenotype without the requirement for co-presentation of self antigen.

FROM TCR ANTAGONISM TO IMMUNE DEVIATION

Central to the aetiology of most experimental autoimmune models is the activation of CD4$^+$ T cells of the Th1 functional phenotype producing pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α).[35] The development of antigen-specific Th1 responses can be counteracted by signals that drive the immune response towards the Th2 pathway.[36] Whilst efforts to control an established autoreactive Th1 response by introduction of Th2 cells have failed,[37,38] this approach has proved successful if the Th2 population is present during the initiation of the Th1 response.[39,40] Further analysis of the effects of the PLP L144/R147 APL revealed more than just TCR antagonism. Priming of 139–151-reactive T cells was not prevented by co-immunization with L144/R147. T-cell clones specific for the APL cross-reacted with native 139–151 but secreted Th2 rather than Th1 cytokines. These Th2 clones were able to suppress EAE when transferred to syngeneic mice and to inhibit encephalitogenic activity of 139–151-speeific T cells *in vitro* prior to adoptive transfer to naïve recipients.[41] Moreover, preimmunization with L144/R147 suppressed EAE on subsequent immunization with different epitopes from PLP, MOG and MBP.[42] This 'bystander suppression' is a powerful tool for treatment of autoimmunity as discussed below.

Why should alteration of the dominant TCR contact residue(s) of a peptide lead to preferential expansion of Th2 cells when using an immunization regime (with CFA) that normally induces Th1 expansion? Studies comparing clones derived from mice immunized with either wild-type 139–151 (Th1) or a Q144 APL (Th2) revealed that Th1 cells primarily recognized residues 143, 144 and 147, whereas Th2 clones recognized residues 141 and 142.[43] Therefore, immunization with APL changed at Th1-binding residues (such as L144/R147) would expand Th2 cells. There have been several reports that the use of APL influence the *in vitro* Th1/Th2 differentiation.[44–46] These presumably reflect different strengths of antigenic signal (different doses of agonist peptide have been reported to effect differentiation: low:Th2; high:Th1: very high:Th2).[47,48] The Q144 studies, however, reveal a unique and intriguing phenomenon: the differentiation of a T-cell population being determined by its fine specificity for antigen. Why this should be in this system remains a puzzle.

Therapeutic use of the ability to convert a pathogenic Th1 response to a benign Th2 response has also been reported in EAE induced with APL derived from the MBP(87–99) sequence in both mice and rats.[49,50] In these studies, exposure of MBP-specific T-cell lines to APL induced a shift in cytokine production with reduction in TNF-α accompanied by increases in interleukin-4 (IL-4). This region of MBP is of particular interest because it is also implicated in the pathogenesis of human MS, being the major MBP epitope recognized by T cells from DR2$^+$ MS patients.[51] Experiments using established human T-cell clones specific for this region identified APL that behaved as TCR antagonists of proliferation and the production of IL-2, IL-4, IL-10 and IFN-γ, but specifically induced *de novo* production of transforming growth factor-β$_1$ (TGF-β$_1$).[31] These APL were therefore switching off production of both Th1 and Th2 cytokines in preference for TGF-β$_1$. Subsequent studies deriving APL-reactive T cells directly from MS peripheral blood revealed APL that inhibited IFN-γ production, allowing selective expansion of IL-4-producing cells.[52]

Co-polymer 1 (also known as Copl, Copaxone, or glatiramer acetate) is a random polymer of alanine, glutamate, lysine and tyrosine with protective effects in several EAE models.[53] Copl is an approved drug for the treatment of MS, reducing the progression of disability and rate of relapse.[53] Investigations of the protective effects of Copl treatment have shown the expansion of myelin-specific Th2 populations in both EAE and MS, indicating immune deviation as a mode of action.[54–56] Copl has also been shown to protect against experimental uveoretinitis[57] and a similar co-polymer inhibited activation of DR4-restricted human T-cell clones specific for type II collagen, a putative autoantigen in rheumatoid arthritis.[58] Although their precise modes of action remain to be defined, random copolymers may therefore provide an approach to more general therapeutic applications.

Taken together these studies provide evidence that: (a) APL can be used to modulate experimental autoimmune models: (b) this may be due to TCR antagonism, immune deviation, or both acting synergistically; and (c) similar phenomena of TCR antagonism and immune deviation can be elicited *in vitro* using human autoreactive T-cell populations.

CAUTION, READ THE SMALL PRINT: THE COMPLEXITIES OF THE AUTOIMMUNE REPERTOIRE

The findings outlined above would argue for the use of APL as TCR antagonists or immune deviators in human autoimmune disorders. Based on our own work, however, it is important to add a note of caution. We have assessed the potential for

TCR antagonism using the MBP(Ac1–9) model of EAE.[15,59] The pathogenic Ac1–9-reactive T-cell repertoire is dominated cells by expressing the Vβ8.2 gene.[60] This model seemed very suitable therefore to test TCR antagonists whose effects may be limited to closely related TCRs. For these studies we used T cells from the Tg4 mouse that expresses a transgenic Ac1–9-specific TCR.[61] As with previously tested Ac1–9-specific cells,[10,14] Tg4 cells recognized residues 6Pro and 3Gln of Ac1–9 as primary and secondary TCR contact residues, respectively. Thus no substitution was permitted at position 6, whereas limited changes (Pro → Met, His, Phe and Tyr) at position 3 could be made without loss of stimulatory activity.[15] Having confirmed position 6 as the primary TCR contact residue, we identified three position 6 APL with potent TCR antagonist activity on Tg4 cells *in vitro*. We next tested these three antagonist APL for encephalitogenic activity *in vivo* (i.e. immunization with individual APL + CFA in the absence of wild-type Ac1–9). As predicted, Tg4 mice developed EAE after immunization with wild-type Ac1–9, but not after immunization with the three antagonist APL. Surprisingly, however, these APL were fully capable of inducing EAE in normal H-2^u mice.[15]

Thus we had the paradoxical findings that APL that inhibited encephalitogenic T cells *in vivo* actively induced disease *in vivo*. The reason for this became apparent when we analysed the fine-specificity of polyclonal Ac1–9-reactive T-cell populations derived from normal H-2^u mice. These cells recognized residues 3 and 6 as primary and secondary TCR contacts, respectively, as the opposite of Tg4 T cells. Importantly, these polyclonal populations were activated by the three position 6 APL that induced EAE in non-transgenic mice. This diverse T-cell cross-reactivity against Ac1–9 APL pointed to considerable complexity in the Ac1–9-reactive T-cell repertoire, in contrast to previous assumptions of an almost monoclonal restriction in TCR usage. This complexity clearly represents a major hurdle to the development of EAE blocking antagonist APL. However, we subsequently identified a position 3 APL that inhibited activation of not only Tg4 cells, but also polyclonal T-cell lines.[59] This APL (with a substitution at the dominant TCR contact residue for the polyclonal response) was an effective inhibitor of EAE when co-immunized with wild-type Ac1–9. Another position 3 APL that only acted as an antagonist for Tg4 cells was a markedly less effective EAE blocker *in vivo*. These results confirm the complexity of the Ac1–9-reactive T-cell repertoire and show that inhibition of the entire population is required for effective prevention of disease.

Our findings in the Ac1–9 EAE model cast doubt on the effectiveness of antagonist APL as therapies in autoimmunity. To identify TCR antagonists we need to generate T-cell clones for *in vitro* analysis. This often results in the dominance of T cells robust enough to withstand the selective pressures of cloning, but that are not representative of the entire *in vivo* repertoire. The Ac1–9 system is analogous to this as the Tg4 TCR was derived from one of a panel of clones showing almost identical TCR usage.[60,61] Designing our antagonist APL on such clones can give a distorted impression of the most suitable peptide to use. Similarly, this may well be the case with APL that induce immune deviation. Thus an APL that converts a Th1 clone to produce IL-4, IL-10, or TGF-β *in vitro* may have the reverse effect on a distinct T-cell clone when given *in vivo*. This unpredictability led us to argue against the use of antagonist or immune deviating APL in human autoimmune disorders.[15] Such an approach in an outbred human population might aggravate rather than reduce pathology.

Reports on two phase II clinical trials in MS patients include results suggesting that this may indeed be the case.[62,63] The effects of APL based on MBP(83–99) were assessed. In one study, although there was no general positive or negative effect of treatment, two of eight patients developed exacerbations associated with APL administration.[62] The APL immunized for T cells producing Th1 cytokines, that cross-reacted with the wild-type MBP sequence and that could be isolated from the cerebrospinal fluid. The second study reported the induction of Th2 responses (in patients receiving the highest dose of APL), as assessed by *in vitro* challenge with either APL or wild-type MBP(83–99).[63] Whilst no overall improvement in clinical parameters was apparent, there was a suggestion of a reduction in development of CNS lesions in patients receiving the lowest dose of APL. This dose of APL did not, however, induce Th2 immunity in those individuals tested. This trial was suspended due to hypersensitivity reactions in a significant proportion of patients, again implying an (over)active Th2 response to APL.

APL-based therapy (at least for TCR antagonism and Th2 immune responses) is therefore complicated by: (a) the complexity of the T-cell repertoire; (b) the unpredictability of the effects of APL; and (c) harmful hyper-reactivity to the APL. It is clear that potential therapy will not be based on a single APL and may even require bespoke drugs for each patient after a rigorous investigation of immune reactivity to modifications of the chosen T-cell epitope.

SUPERAGONISTS AND APOPTOSIS

If the use of antagonist or immune-deviating APL is problematic, is there a better approach? What do we want to do to the autoreactive T cells driving the pathology? Perhaps the safest outcome is to kill them. It is well established that CD4$^+$ T cells, once activated are highly sensitive to deletion via activation-induced cell death. High doses of antigen can lead to apoptosis of antigen-specific T cells *in vivo*.[64,65] This 'proliferation' cell death has been proposed as a major homeostatic mechanism that shapes the immune repertoire by removal of 'overstimulated' T cells.[66] If the apoptotic signal is driven by the strength of signalling through the TCR, then a dose of wild-type antigen be replaced by using lower doses of superagonist APL. The MBP(Ac1–9) model allows us to address this due to the range of APL available with well-defined antigenic properties *in vitro*. We can assume that T cells primed against wild-type Ac1–9 must express high-affinity TCRs to compensate the peptide's vanishing affinity for A^u and allow formation of a productive MHC:peptide:TCR complex.

Immunization with Ac1–9 expands these highly sensitive T cells required to induce EAE. Paradoxically however, superagonists failed to prime effectively for EAE induction.[15,20] We analysed the *in vivo* fate of the highly sensitive T cells by transferring fluorescently labelled naïve Tg4 T cells into non-transgenic recipients prior to immunization with either wild-type Ac1–9 or the 4Tyr superagonist.[20] After 4Tyr immunization more than 70% of Tg4 cells showed signs of apoptosis compared with less than 10% in response to

wild-type Ac1–9. Highly sensitive Ac1–9-reactive cells therefore undergo negative selection (through antigen-induced cell death) on *in vivo* exposure to superagonist ligands.

This provides an explanation for the early results that co-immunization of Ac1–9 with the 4Ala superagonist failed to induce EAE.[13] Highly sensitive T cells would be deleted on 4Ala before they had a chance to initiate an inflammatory lesion in the CNS. Peripheral deletion also explains the failure to transfer protection using T cells from 4Ala-primed mice.[21] Apoptosis in response to superagonist stimulation has also been shown *in vitro* using an independently derived Ac1–9-specific TCR transgenic mouse.[67] Similar *in vivo* findings have been reported using a superagonist APL in the MBP(87–99) EAE model.[68] Antigen-induced cell death has also been shown using relatively low doses of an oligomerized form of influenza virus haemagglutinin peptide.[69] The development of 'killer APL' may therefore provide a therapeutic avenue for human diseases. One drawback would be that superagonists generally induce a cytokine burst prior to apoptosis. Such a burst, although hopefully only transient, might lead to a significant clinical exacerbation. It is encouraging to note therefore that APL have been developed that are capable of inducing apoptosis in activated T cells specific for pigeon cytochrome c, but without the production of cytokines seen in association with apoptosis induced by native peptide.[70]

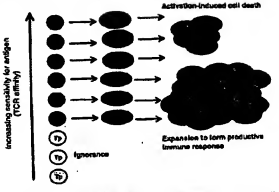


Figure 3. An avidity model for clonal selection. The naive T-cell repertoire will contain a heterogeneous population of potentially antigen-reactive T-cell precursors (Tp) expressing diverse TCRs. These TCRs will have a range of affinity for the antigen. T cells with low-affinity TCRs (and therefore low sensitivity) for antigen will remain unaffected by exposure to antigen (via infection, immunization, or exposure of self antigen during inflammation/tissue damage). T cells bearing TCRs with high affinity will become activated but are speedily deleted from the repertoire by apoptosis. Only T cells expressing TCRs with 'moderate' affinity will be activated without signals for apotosis and so can expand and form the productive immune response. This serves to maintain T-cell sensitivity for the inducing antigen within a predetermined (and as yet undefined) 'window'.

AN AVIDITY MODEL FOR CLONAL SELECTION

Our studies of Ac1–9 superagonists revealed a further striking feature.[20] T-cell populations generated by immunization with Ac1–9 or any of the APL all showed identical sensitivity to the antigen they were originally primed against (with responses first apparent at ~10 nanomolar). Staining antigen-reactive T cells with fluorescently labelled peptide:MHC class II tetrameric complexes provides a measure of TCR affinity for that complex.[71,72] Using tetrameric A^u:Ac1–11 complexes we determined that the T cells that avoid deletion after immunization with superagonist expressed distinct (not Vβ8), low-affinity TCRs compared to the pathogenic T cells expanded by wild-type Ac1–9. Thus T-cell lines generated against Ac1–9 were heterogeneous populations containing cells expressing low-, moderate- and (around 50%) high-affinity TCRs. T cells generated against the 4Tyr superagonist, in contrast, only used low-affinity TCRs. This low affinity explains why superagonist-primed T cells are relatively insensitive to superagonist, responding with the same kinetics to superagonist as Ac1–9-primed cells respond to Ac1–9.

These results led us to propose an avidity model for clonal selection during peripheral immune responses to antigen (summarized in Fig. 3).[20] This model argues that due to the high level of TCR cross-reactivity for antigen,[73] the immune repertoire will contain a heterogeneous population of T cells capable of antigen recognition with sensitivities ranging from low to high. Relatively insensitive T cells will not receive a strong enough stimulus and will remain ignorant of the presence of antigen. At the other extreme, T cells that are too highly sensitive (due to having high-affinity receptors) will undergo antigen-induced cell death. Only T cells displaying sensitivity for the priming antigen within a defined range will therefore be allowed to undergo successful clonal expansion and constitute the productive repertoire. This process would explain why we and others consistently generate antigen-specific T cells with this constricted sensitivity for the priming antigen. Such efforts of avidity are of course not new. They form the basis for our perception of positive and negative selection during thymic development.[74] What our results tell us is that mature T cells are also under avidity-based control once in the periphery. We have described the constriction of T cells into the productive sensitivity window as 'tuning'. We use this word to describe the effects on the entire antigen-reactive repertoire via TCR-based selection. This is distinct from tuning at the single cell level which presumably involves rewiring of components of the cell signalling machinery.[74–76]

ARE CHANGES NECESSARY? FROM APL TO UNALTERED PEPTIDES AND BACK AGAIN

Perhaps the best therapeutic approach is to induce autoantigen-specific T-cell tolerance using native antigen. It has been evident for some 40 years that the form in which antigen is administered has a dominant influence on the decision whether to mount a response or not.[77] Antigen in aggregated form, or mixed with adjuvant, will provoke an overt response, whereas soluble monomeric antigen induces a state of antigen-specific T-cell tolerance. We and others have given soluble peptides via the oral, intravenous, intraperitoneal, or intranasal routes.[78–80] This approach can effectively inhibit T-cell activation in response to antigen in adjuvant. Moreover, it has proved effective in inhibiting the onset of disease in EAE[78–80,83,84] as well as experimentally induced models of arthritis,[85,86] uveoretinitis[87] and MG.[80] In addition, administration of

antigenic peptides from insulin or glutamate decarboxylase (GAD) 65 can inhibit the spontaneous development of diabetes in the non-obese diabetic (NOD) mouse.[84,89] The exact mechanism underlying tolerance induction with soluble peptides remains an issue of debate and has been proposed to involve the induction of apoptosis, anergy, or a regulatory phenotype in antigen-specific T cells.[90] Tolerance may in fact involve all of these processes with different cells being killed, energized or diverted to regulatory function during the tolerance process.

The paucity of knowledge of the T-cell epitopes that are critical to pathogenesis raises an obstacle to the use of peptide-induced tolerance in human autoimmune disorders. For certain diseases the autoantigens are relatively well defined, such as the AChR in MG[91] and thyroglobulin and thyroperoxidase in autoimmune thyroid disease.[92] For other diseases, candidate autoantigens have been proposed but we cannot definitively assess their role in disease or rule out the contribution of other as yet undefined autoantigens. Recent evidence using 'humanized' mice have revealed that the immunodominant MBP83–99 epitope, which is implicated in MS pathogenesis, can indeed elicit EAE.[93] Other epitopes have yet to be assessed in this way, however. A further complicating factor is epitope spreading,[94] a mechanism by which autoreactivity spreads to new epitopes due to tissue damage as disease progresses. This not only increases the number of epitopes recognized, but precludes identification of the epitope(s) involved in initiation of autoimmune attack once the clinical signs have developed, making prophylaxis difficult.

The question facing peptide-based tolerance, therefore, is how can we tolerize T cells against all known and unknown epitopes involved in disease? The answer may lie in the induction of bystander suppression. By this we mean the suppression of responses to multiple epitopes after administration of a single epitope. This phenomenon has been described using the *Der p* I house dust mite allergen as the model antigen. Intranasal administration of the immunodominant epitope of *Der p* I resulted in tolerance to this epitope and subdominant epitopes upon subsequent immunization with the intact protein.[81,82]

We tested the ability of three encephalitogenic peptides to induce suppressive effects in EAE: Ac1–9 and 89–101 of MBP and PLP(139–151).[80] Intranasal administration of Ac1–9 suppressed responses to both Ac1–9 and 89–101 on subsequent immunization with intact myelin, but did not suppress responses to PLP(139–151). The PLP epitope proved an even more effective tolerogen, suppressing responses to itself and both MBP epitopes. So bystander suppression could be induced at the level of T-cell priming in the draining lymph node. Intranasal administration of a single peptide did not block T-cell priming but could suppress EAE after immunization with a different peptide. Thus PLP(139–151) suppressed EAE induced with itself or either of the two MBP epitopes, whereas Ac1–9 only suppressed disease induced with each of the MBP peptides but not the PLP peptide. Figure 4 supplies a model for bystander suppression that can function in either the lymph node draining the site of immunization with whole myelin or in the lymph nodes draining the CNS after immunization with peptide. The increased effectiveness of the PLP epitope over the MBP epitope may well reflect a comparatively higher frequency of PLP(139–151)-specific

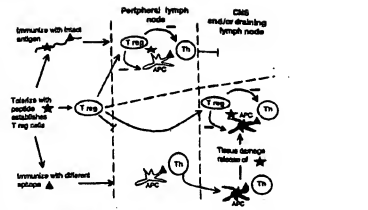


Figure 4. Mechanism for bystander suppression active either in the periphery or site of autoimmune attack. Induction of tolerance with soluble peptide leads to the expansion of an epitope-specific T regulatory (T reg) cell population. Subsequent immunization with intact antigen will lead to presentation of this epitope (red star) in addition to other pathogenic epitopes to which tolerance has not been induced (green triangle) in the draining lymph nodes. This leads to recruitment of pathogenic T cells capable of inducing disease. However, T reg cells are also recruited and suppress activation of the pathogenic T cells either directly, or indirectly via regulation of APC function. Immunization with only the epitope against which the immune system has not been tolerized will fail to recruit T reg cells, therefore allowing the pathogenic T cells to be fully activated. These cells then migrate to the target organ (in this case the CNS) and initiate an inflammatory lesion. Subsequent tissue damage releases the epitope recognized by the T reg cells which then are recruited to the target organ and/or lymph nodes draining the target organ where they can now exert their suppressive effects.

precursors in naive mice due to a lack of thymic expression of this region of PLP.[95] Thus, a high frequency of tolerant PLP-reactive cells could suppress a low frequency of MBP-reactive cells. A low frequency of tolerant MBP-reactive cell may not however, suppress a high frequency of PLP-reactive cells.

Bystander suppression has also been reported in other studies of administration of soluble myelin-derived peptides in EAE.[83,96] These studies, coupled with those in the *Der p* I model[81,82] and the L144/R147 APL of PLP described earlier,[97] present a persuasive argument that the induction of active suppression using soluble peptides will be the most effective therapeutic approach.

The precise nature of the suppressive effects is an area of controversy. In the NOD mouse soluble peptides have been reported to drive the immune response towards a Th2 phenotype.[84,89] T cells expanded as a result of oral tolerance have been reported preferentially to secrete TGF-β (the so-called 'Th3' phenotype).[98] We were unable to detect a shift towards Th2 or Th3 immunity in normal mice.[80] Studies in Tg4 transgenic mice, however, demonstrated down-regulation of IL-2, IL-4 and IFN-γ production, but up-regulation of IL-10 production following peptide treatment.[97] Furthermore, the administration of a neutralizing anti-IL-10 antibody restored susceptibility to EAE in peptide-treated Tg4 mice. The immunosuppressive properties of IL-10 have been implicated in control of pathology in EAE.[99,95] T cells that preferentially

express IL-10 (T reg or Tr1 cells) have also been shown to have potent suppressive effects in models of inflammatory bowel disease.[99]

A note of caution to the systemic application of peptides comes from a recent description of anaphylaxis in EAE models.[100] EAE was induced with either PLP(139–151) or MOG(35–55). Anaphylaxis and death resulted when the same peptides were given in solution intraperitoneally after disease resolution (but not when given earlier during active disease). This may reflect a Th2 response associated with the later remission stages of EAE. Indeed, in our studies repeated administration of the PLP peptide after onset of EAE induced with whole myelin led to a significant reduction in incidence and severity of relapses without signs of anaphylaxis.[87] This relapsing form of disease induced with myelin may not involve a switch to Th2. Immunization with peptide may well induce a significantly larger antigen-reactive T-cell population, many of which may play no role in pathogenesis due to recognition of the peptide in a form not generated by processing of native antigen. We have evidence that immunization with MOG(35–55) expands a heterogeneous population of cells of which only a fraction respond to intact MOG and it is only these MOG-reactive cells that transfer EAE (S.M.A. unpublished observations). Anaphylaxis in the peptide-induced models may thus be the result of reactivation of cells not directly involved in disease. This may not therefore be directly relevant to the human situation in which autoreactive T cells will be activated by presentation of processed self antigen rather than by immunization with a high dose of synthetic peptide.

It is worth noting that there are situations in which we might be able to improve the efficacy of soluble peptide therapy by making changes to the antigen sequence. Hence APL may after all be an attractive approach. Firstly, improving the affinity of a peptide for MHC creates a stronger tolerogen.[78,79,84] Wild-type MBP(Ac1–9) is relatively poor at inducing tolerance compared to the position 4 superagonists with increased affinity for class II. Our experiments showing bystander suppression therefore used the 4Tyr APL to induce tolerance.[89]

In our studies of the suppressive effects of MBP and PLP peptides we observed that the MBP(89–101) peptide failed to prevent EAE induced even with itself.[85] Thorough analysis reveals complex T-cell responses to this peptide (S.M.A. manuscript in preparation). There appears to be three overlapping epitopes within this short 13mer peptide recognized by distinct T-cell populations. Two of these epitopes are generated from antigen processing of native MBP whereas the third epitope is 'cryptic', i.e. it is not generated form intact MBP.[101] Peptides containing the two naturally processed epitopes are capable of inducing EAE, whereas the cryptic epitope is not. This presumably reflects a requirement for autoreactive T cells to recognize epitopes processed from native MBP in the CNS. T-cell lines derived from MBP(89–101)-immunized mice show preferential recognition of the cryptic epitope, suggesting that this is the dominant configuration assumed by the class II:89–101 complex. Thus the encephalitogenic T-cell population constitutes only a minor fraction of the total 89–101-reactive T-cell pool. Furthermore soluble application of 89–101 appears to tolerize T cells recognizing the dominant cryptic epitope, but not those recognizing the minor disease-relevant epitopes. This would account for the failure to prevent EAE when tolerizing with this peptide. One potential way to make this peptide tolerize to EAE would be to generate APL with appropriate substitutions that ablate the formation of the cryptic peptide:class II conformation. The disease-relevant epitopes should then become dominant and capable of delivering the tolerance signal to the pathogenic T cells. Such an approach using APL would minimize the risk of anaphylaxis due to expansion of high numbers of T cells responsive to cryptic epitopes as described above. For instance, in the MOG(35–55) system we are currently exploring APL that preferentially stimulate those T cells that are responsive to intact MOG and induce EAE. These APL would be ignored by 35–55-reactive cells that are not relevant to disease. Indeed it may well be that tolerizing these cells would be undesirable as they might perform useful functions due to cross-reactive recognition of foreign antigens not related to autoimmunity.

Another potential area for the use of APL involves T-cell recognition of antigens that have been post-translationally modified. Such antigens may be present in the target organ but not in the thymus, allowing the escape from central tolerance of T cells that specifically recognize the post-translationally modified form of the antigen. Thus collagen-induced arthritis has been demonstrated to involve T-cell recognition in glycosylated epitopes.[102] More recently, dominant T-cell responses to α-gliadin in coeliac disease have been shown to require the conversion of glutamine residues to glutamate via the action of tissue transglutaminase.[103] Such enzyme-mediated alterations in antigen sequence may have important roles in the activation of autoaggressive T cells. The use of APL may therefore improve not only the identification of autoantigenic epitopes, but also our attempts to control the activities of self-reactive cells.

CONCLUDING REMARKS

The use of APL over the past decade has formed a central plank in our efforts to probe the molecular requirements for T-cell activation. Identification of APL that act either as TCR antagonists or immune deviators prompted their successful use in animal models of autoimmunity. These experiments, however, have also highlighted the considerable complexity and diversity of the self-reactive T-cell repertoires in what first appeared to be simple systems. These complexities will be greater in the outbred human population and require extreme caution when designing APL-based therapies as highlighted by recent clinical trials.

A more favourable approach therefore is the use of natural peptide sequences to tolerize the immune system, preferably by expansion of self-reactive T regulatory cells capable of bystander suppression. Even this approach may need to be improved, however, providing new avenues for the application of APL to allow: (a) the focusing tolerance on those cells relevant to disease; (b) the avoidance of hypersensitivity reactions to cryptic epitopes within the drug; and (c) investigation of the role of modified self antigens in autoimmune pathogenesis.

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EXHIBIT H

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Transplantation tolerance: The concept and its applicability

Dong VM, Womer KL, Sayegh MH. Transplantation tolerance: The concept and its applicability.
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Victor M. Dong*, Karl L. Womer* and Mohamad H. Sayegh
Laboratory of Immunogenetics and Transplantation, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA

Key words: alloresponse – costimulatory blockade – rejection – tolerance – transplantation

Mohamad H. Sayegh, Laboratory of Immunogenetics and Transplantation, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, Massachusetts, 02115, USA
Tel: (617) 732-6253
Fax: (617) 732-6254
E-mail: msayegh@rics.bwh.harvard.edu

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Abstract: Recent advances have enabled researchers to induce tolerance in animal transplant models. Although it has been relatively easy to do so in rodents, it has been much more difficult to translate such strategies into primates. Understanding the cellular and molecular mechanisms of the alloimmune response has prompted the development of novel strategies that may obviate the need for immunosuppression in humans. Mechanisms of tolerance and promising new therapies, as well as the inherent difficulties in bringing them into clinical practice, are reviewed.

With improvements in surgical techniques, infectious disease therapy and overall medical care over the past few decades, manipulation of the immune response remains the major barrier to successful organ transplantation. The introduction of cyclosporin A into clinical use in the early 1980s (1) and the development of newer immunosuppressive drugs (2) have led to a significant reduction in acute rejection rates and improvement in short-term allograft survival. However, achieving long-term graft survival and overcoming chronic rejection remain difficult tasks (3, 4). Moreover, these drugs cause non-specific immunosuppression and result in an increased risk of infection, malignancy and cardiovascular disease. Therefore, the major goal of transplantation research is the development of strategies to induce donor-specific tolerance.

Tolerance has been defined as a state of specific immunologic unresponsiveness to the antigens of the graft in the absence of maintenance immunosuppression (5). However, it is now clear that active immunoregulatory mechanisms may be important in the development and maintenance of tolerance. Perhaps a more accurate definition of tolerance would be the absence of a destructive immune response against the graft in an immunocompetent host (6). *In vivo* criteria for donor-specific tolerance are the absence of acute rejection with prolongation of graft survival and acceptance of second test grafts from the original donor, while maintaining the ability to reject third party grafts. Unfortunately, investigators have only recently started to examine graft morphology and function in long-term surviving 'tolerant' animals. This is important, because long-term graft survival does not necessarily imply tolerance. In fact, tolerance should not only prevent acute rejection but also the alloantigen-dependent component of chronic allograft dysfunction, the major cause of late graft dysfunction and loss in solid organ transplantation (7).

Abbreviations: Ag, antigen; APC, antigen presenting cell; CD, cluster of differentiation; CTLA, cytotoxic T lymphocyte antigen; FasL, Fas ligand; gp39, glycoprotein 39; ICAM, intercellular adhesion molecule; ICOS, inducible co-stimulator; Ig, immunoglobulin; IL, interleukin; IL-2R, interleukin-2 receptor; IFN, interferon; LFA, leukocyte function associated antigen; MHC, major histocompatibility complex; TCR, T-cell receptor; Th, T helper; TNF, tumor necrosis factor.

* Co-first authors. This material was presented at the 3rd International Congress on Pediatric Transplantation, Boston, MA, USA, July 1998.

Billingham et al. in 1953 were the first to describe a state of actively acquired 'immunologic tolerance' in mice that had been injected *in utero* or during the neonatal period with bone marrow derived donor cells (8). Such animals would later accept an allograft taken from the same inbred strain from which the original cells were harvested, while maintaining the ability to reject third party strain grafts. Since these observations, extensive research has focused on developing strategies to understand the mechanisms of tolerance and to induce tolerance in adult animals. The ultimate goal would be to translate this research into large animals and eventually humans. Although it has been relatively easy to induce tolerance in rodents, it has proven to be much more difficult to translate such strategies into primates. Nonetheless, recent advances in our understanding of the cellular and molecular mechanisms of the alloimmune response have fueled the development of promising novel strategies that may be successfully translated into humans (5, 9, 10). The purpose of this review is to provide a brief overview of the mechanisms of tolerance, to describe some of the promising novel strategies that may be translated into humans, and finally to summarize the major problems we are facing in developing strategies to induce tolerance in humans.

Mechanisms of tolerance

Before discussing the current strategies designed to induce tolerance in the transplant recipient, it is worth reviewing the basic mechanisms of tolerance (11). Immunologic tolerance may be mediated by thymic deletional mechanisms (central tolerance) or be induced/maintained throughout the peripheral blood and extrathymic lymphoid tissue (peripheral tolerance). by at least three different mechanisms: deletion, anergy or regulation/suppression. These mechanisms will be reviewed as they relate both to self- and transplantation tolerance.

Self-tolerance

The physical elimination of certain T cells that interact with self-MHC molecules within the thymus plays an important role in the development of tolerance to self-antigens in the fetal/neonatal period (12). Phenotypic expression of T cells is determined by the sum of random TCR gene rearrangements (13). T-cell precursors originate in the bone marrow and migrate to the thymus where they initially fail to express the CD4 and CD8 T cell markers that are associated with maturity. These *double-negative* T cells then undergo proliferation and maturation and acquire both CD4 and CD8 T cell markers to become so-called *double-positive* cells. Through random TCR gene rearrangements, these thymocytes express TCRs with varying affinity for self-MHC + peptide.

Central to the mechanisms of self-tolerance is the avidity with which the TCR interacts with the self-MHC + peptide. This is determined by both the structure of the TCR for the antigen as well as the density of TCRs present on the T cell (14, 15). Thymocytes that either lack affinity or have very high affinity for the complex are negatively selected and undergo deletion by programmed cell death (apoptosis). Those thymocytes with low avidity for the complex are positively selected and become a set of T cells that function with self-MHC but lack sufficient autoreactivity to result in autoimmune disease. Some lower avidity TCRs may escape at this stage only to be deleted later with the increase in TCR expression that occurs with the transition from double positivity ($CD4^+$ /$CD8^+$) to single positivity ($CD4^+$ /$CD8^-$ or $CD4^-$/$CD8^+$) as thymocytes migrate from the cortex to the medulla of the thymus. Positively selected medullary thymocytes acquire MHC class II restricted helper ($CD4^+$) or MHC class I restricted cytotoxic ($CD8^+$) functions and migrate to the periphery as immunocompetent T cells. Between 95 and 99% of thymocytes are deleted in the thymus, with only the small remainder becoming mature T cells that migrate to the periphery. It is thought that autoimmunity arises when some autoreactive T cells escape thymic negative selection.

There are two types of thymic APCs: bone-marrow-derived macrophage/dendritic cells and epithelial cells (16). There is evidence that each type has a different function in the induction of self-tolerance and in T-cell repertoire selection. Positive selection is thought to be mediated by thymic epithelial cells, whereas negative selection is thought to be mediated by bone marrow-derived cells. Since the thymus involutes after puberty, clonal deletion may play only a minor role in the development of tolerance in the adult. However, cells that are important in the induction of thymic (central) deletional tolerance may still be functional. In addition, there is some evidence that T-cell death occurs in the periphery, assisting with the maintenance of self-tolerance and prevention of autoimmunity and possibly playing a role in transplantation tolerance.

Transplantation tolerance (Table 1)

Induction of tolerance in adults involves several mechanisms that are best classified as central or peripheral. Central tolerance involves *thymic deletional* mechanisms analogous to self-tolerance and can be induced in experimental animals by creating bone marrow chimeras (17). These chimeras are achieved by donor bone marrow infusion into a recipient treated with a myeloablative regimen such as total body irradiation. Donor APCs from this inoculum migrate to the thymus of the reconstituted animal, where they will be seen as *self*. Thereafter, the resultant animal will be specifically tolerant to donor alloantigen, presumably by deletion of alloreactive T cells in the thymus (negative selection).

Besides the potential complication of graft-versus-host disease, which can be prevented by T cell depletion of the bone marrow preparation, chimeric animals may have deficient T cell responses to nominal antigens. As previously mentioned, T cells are positively selected during normal maturation to recognize foreign antigens in association with the self-MHC molecules expressed on thymic epithelium. In these chimeras, however, the bone marrow-derived APCs are now of donor origin, whereas the thymic epithelial APCs remain of recipient origin. As a result, mature T cells will be positively selected to respond only to antigens presented by the recipient MHC molecule but not to antigen presented in association with donor-MHC on APCs derived from the donor bone marrow. Consequently, the T cell immune response is suppressed (17).

This problem can be overcome by creating mixed allogeneic chimeras by reconstituting the myeloablated animals with a mixture of both syngeneic plus allogeneic bone marrow (18). The reconstituted animals have a mixture of APCs derived from both recipient and donor and therefore have normal T cell function. Such a strategy has been successful in both rodents and non-human primates, in allo- as well as xeno-transplant models (17). However, the clinical applicability of such strategies is questionable because of the potential for major side-effects. Non-myeloablative regimens such as donor bone marrow infusion with administration of antilymphocyte serum or other immunosuppressive drugs have been used (19), although the mechanisms involved with such strategies may not be all deletional (20).

Mechanisms of peripheral tolerance generally involve *anergy* and/or *regulatory/suppressor cells*, although *peripheral deletional* mechanisms probably also play a role. One possible mechanism leading to tolerance that has been described with donor bone marrow infusion strategies is *microchimerism* (21, 22). Microchimerism is the persistence of small numbers of donor-derived cells in the recipient and has been reported to be associated with long-term acceptance of allografts in experimental animals as well as in humans (23). However, it has not yet been established whether the persistence of donor cells is actually responsible for the induction or maintenance of tolerance. The observation by Fraser et al. that the donor bone marrow can be replaced with recipient marrow transfected with donor MHC indicates that microchimerism is not necessary for graft acceptance and suggests that a supply of donor antigens for presentation by the indirect pathway of allorecognition (i.e. by recipient APCs) may be tolerogenic (24). The distinction between the indirect and direct pathways will be discussed later in more detail.

Veto cells are cells with a unique phenotype that have been shown to inactivate/delete alloreactive T cells. These cells have been described in some transplantation models involving donor bone marrow infusion (21, 22), although the mechanisms of how T cells recognize the veto cell leading to T cell inactivation/deletion remain unknown. Furthermore, whether veto cells mediate the observed association between graft acceptance and microchimerism has not been established.

Anergy is a state of functional inactivation in which antigen-specific T lymphocytes are present but unable to respond. Unresponsiveness can be assessed *in vitro* by failure of proliferation and cytokine production (25) and *in vivo* by failure of clonal expansion (26, 27). Two types of anergy-related mechanisms have been described: 1, T cell anergy, which may be reversed by exogenous cytokines such as IL-2 (28, 29); and 2, *donse* anergy, which is not reversed by cytokines (30). Recent evidence also indicates that anergy can be accompanied by variable degrees of deletion and that anergic T cells may become apoptotic (31). From the standpoint of clinical applicability, strategies that promote peripheral deletion of anergic T cells are more desirable. In this regard, there has been recent interest in studying the role and mechanisms of activation induced cell death in induction and maintenance of transplantation tolerance (32, 33).

It is now clear that T cells require two distinct signals for full activation. The first signal is provided by the engagement of the TCR with the MHC + peptide complex on APCs, and the second *costimulatory* signal is provided by

engagement of one or more T cell surface receptors with their specific ligands on APCs. TCR ligation in the absence of additional signals provided by the MHC-expressing APC results in antigen-specific unresponsiveness or anergy (34). While several receptor-ligand interactions have been suggested to provide costimulatory signals to T cells (CD2: LFA-3, LFA-1: ICAM-1), the best characterized costimulatory signal involves binding of the T cell surface molecule CD28 to either of its ligands, B7-1 or B7-2, expressed on the surface of professional bone marrow derived APCs (see Fig. 1).

The uniqueness of this costimulatory pathway has been demonstrated clearly in studies indicating that signaling through CD28 prevents T cell anergy and death induced by TCR signaling alone (35, 36). CD28 has a high degree of homology (32% identity at the amino acid level) to another gene called CTLA4 (37). Unlike CD28, which is expressed on resting T cells, CTLA4 appears to be expressed on the cell surface only after initial T cell activation. CTLA4 appears to downregulate immune responses by binding with high affinity to its B7 counterreceptors (38, 39). CTLA4 gene knockout mice show a variety of lymphoproliferative disorders and early death (40), while administration of blocking anti-CTLA4 monoclonal antibodies (to block negative T cell signaling) worsens autoimmune disease (41) and prevents induction of peripheral tolerance (42), implying a critical physiologic role for CTLA4 in terminating T cell responses (43).

Another means by which tolerance is regulated is through the induction of specific regulatory or *suppressor* cells. These cells have been demonstrated *in vitro* by suppressor assays as well as *in vivo* by adoptive transfer experiments that may lead to a state of 'infectious' tolerance (44, 45), whereby T cells from a tolerant animal may actively transfer tolerance to a naive animal. Although suppressor phenomena have been clearly demonstrated, the suppressor cells themselves have been difficult to clone and, hence, to characterize. In addition, the mechanism of action of suppressor cells is still poorly understood, although recent data suggest that the tolerogenic effects may be mediated by suppressive cytokines (46).

A related area which may explain the role of these regulatory cells in tolerance is the controversial $T_{H}1/T_{H}2$ paradigm (47–50). It has been recognized that $CD4^{+}$ T cells can be subdivided into T helper 1 ($T_{H}1$) and T helper 2 ($T_{H}2$) subsets that are classified according to the profile of cytokines they secrete. $T_{H}1$ cells secrete IL-2 and IFN-γ and predominately mediate cellular immune responses, while $T_{H}2$ cells secrete IL-4, IL-5, IL-10 and IL-13 and regulate humoral immune responses. $T_{H}1$ cytokines have been shown to be upregulated in rejecting allografts, while $T_{H}2$ cytokines have been shown to be expressed in 'tolerant' grafts (51, 52). Such a state of immune deviation towards predominately $T_{H}2$ cell function has been associated with tolerance in models of transplantation, although the causality of such an association has not been proven. Indeed, recent evidence in specific cytokine gene knockout animals showing that $T_{H}1$ knockout mice can reject a graft (53) and that $T_{H}2$ knockout animals can be tolerized (54) illustrates the complexity of the system.

Novel clinical strategies

The major shortcomings of modern transplantation medicine are the consequences of lifelong immunosuppression. It would be more desirable to achieve a state of immunologic tolerance, which would obviate the need for immunosuppression. This challenge is heightened by the fact that immunosuppressive agents may actually abrogate the induction of tolerance (55–57). Nevertheless, there are a number of promising strategies currently being investigated.

Intrathymic tolerance

The initial studies in the 1960s of intrathymic inoculation of soluble antigen into adult rats demonstrated antigen-specific systemic T cell unresponsiveness. It is now recognized that this unresponsiveness is probably achieved via anergy and cell deletion (apoptosis) (58). Intrathymic injection of antigen (Ag) induces Ag-specific tolerance in several experimental autoimmune and transplantation models. However, the clinical applicability of thymic injection of alloantigen still requires further investigation (59, 60).

Central tolerance inducing strategies that manipulate T cell events in the thymus have involved lethal or sublethal myeloablation followed by immune response reconstitution with a combination of donor and recipient bone marrow, as mentioned earlier (61). Another approach uses transfected recipient bone marrow cells with donor MHC, obviating the need for donor bone marrow cells. In an effort to reduce the toxic irradiation dose to large animals, synergistic therapies such as polyclonal/monoclonal antilymphocyte antibodies, cyclophosphamide or costimulatory blockade have been or are being tested. This approach allows for host bone

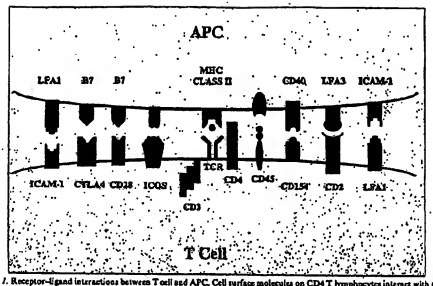

Fig. 1. Receptor-ligand interactions between T cell and APC. Cell surface molecules on CD4 T lymphocytes interact with their respective ligands on the APC. Interactions between these molecules are important in cell–cell adhesion and/or transmission of signals (including costimulatory signals) to immune cells.

marrow suppression with minimal side-effects, resulting in stable mixed chimeric animals. These models have shown promise in non-human primates as a preclinical strategy (62).

Donor bone marrow infusion

The use of donor bone marrow augmentation utilizes aspects of both central and peripheral tolerance (23). Donor bone marrow is injected into patients at the time of solid organ transplantation, with subsequent low level persistence of donor cells leading to a microchimeric state. As discussed previously, long-term allograft function in humans and animals is often associated with persistence of donor-derived hematopoietic cells in the recipient. However, it is unclear whether donor cells persist due to immunosuppression or if they are themselves responsible for prolonged long-term graft survival. Barber et al. examined the use of Minnesota antilymphocyte globulin and subsequent transfusion of donor-specific bone marrow in 57 cadaveric renal allograft recipients. When compared with controls, donor-specific bone marrow infusion was associated with improved allograft survival (63). More recently a clinical trial looked at the effect of timing and dose of peripheral donor bone marrow cell infusion on graft and patient survival after liver transplantation (64). As with the renal allograft recipients, graft survival was significantly improved in the treatment group. The optimal utility of bone marrow infusion/augmentation has not been defined in multicenter, randomized clinical trials.

Immunomodulatory peptides

Observations that some peptides bound to MHC molecules are derived from MHC sequences themselves has prompted the study of the immunoregulatory properties of MHC derived peptides. In transplantation, two pathways of allorecognition are recognized (10, 65–67). The direct pathway involves recipient T cells recognizing intact allo-MHC molecules complexed with peptide on the surface of donor cells. The indirect pathway involves recipient APCs processing and presenting alloantigens (mainly allo-MHC peptides) to recipient $CD4^+$ cells (see Fig. 2). In the latter situation, T cell responses to an alloantigen may be limited to one or only a few dominant peptide determinants. Therefore, tolerizing to these peptide determinants alone may then successfully inhibit at least indirect T cell alloresponses (68).

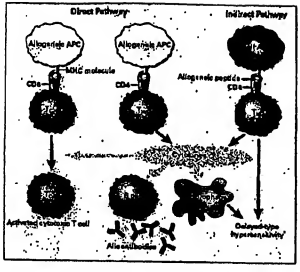

Fig. 2. Pathways of recognition of allogeneic MHC molecules and mechanisms of graft rejection. Graft rejection is usually initiated by CD4 helper T cells (T_H) that bind peptides in complexes with MHC class II molecules on antigen-processing cells. In the direct pathway, an MHC molecule on a foreign (allogeneic) cell, such as an APC, binds to the helper T cell. In the indirect pathway, the foreign MHC molecule is processed into peptides that are presented to the helper T cell by one of the recipient's own APCs. In either scenario, activated CD4 helper T cells proliferate and secrete cytokines that serve as growth/activation factors for CD8 cytotoxic cells (T_C), B cells and macrophages. These cells in turn cause graft destruction by target cell lysis, antibody production and delayed-type hypersensitivity reactions, respectively. [Reprinted with permission: N Engl J Med 1998;338:1813–21. © 1998 Massachusetts Medical Society. All rights reserved.]

Strategies designed to induce allogeneic unresponsiveness using peptides derived from both polymorphic and non-polymorphic portions of class I and II MHC have been studied (69). Boytim et al. were able to block allo-non-specific induced T cell proliferation with a synthetic peptide corresponding to the alpha helical region of HLA-DQ by interrupting cell-cycle progression (70). Similarly, Murphy et al. were able to alter T cell alloimmune responses *in vitro* with synthetic peptides derived from a highly conserved region of the class II MHC alpha chain through the induction of apoptosis (71). *In vivo* studies have demonstrated reduced delayed-type hypersensitivity responses to a mixture of polymorphic class II MHC allopeptides in peptide immunized rats with oral administration of the peptide mixture prior to immunization (72). Since indirect allorecognition may play a role in chronic rejection, synthetic MHC peptides may also be used in the future to develop predictive assays that link T cell activity to the subsequent risk of developing chronic rejection post-transplant (73, 74). Whether tolerizing to MHC peptides, by oral administration for example, will have an impact on delaying progression of chronic rejection remains to be seen.

Other therapies

The targeting of T cell epitopes by monoclonal antibodies is currently undergoing intense investigation. The humanized monoclonal antibodies, daclizumab and basiliximab, are targeted against the 55Kd alpha subunit of the IL-2 receptor (2) and have recently been approved by the FDA for use as induction therapy in clinical renal transplantation (75). However, their role in the induction of tolerance has not yet been established.

There has been an increased understanding of the molecular interactions between the T cell and APC, as discussed previously. Illustrated in Fig. 1 are some of the better characterized receptor–ligand couplings (2). Antibodies directed against some of these cell surface molecules have been used in experimental small animal models to induce tolerance (76). The clinical applicability of some of these strategies is currently being tested.

Newer approaches still in their infancy include gene-targeted therapies (77). For example, it has been demonstrated in the mouse diabetic model that FasL expressed in syngenic myoblasts, made by transfection of myoblasts of recipient origin, can protect allogeneic islets of Langerhans from rejection, presumably through Fas/FasL-induced apoptosis of reactive T cells (78). In clinical transplantation, gene therapy may enable immunomodulatory agents to be expressed in the graft, thereby overcoming the difficulties of systemic immunosuppresion (2).

T-cell costimulatory blockade

Although there are several experimental strategies that may hold promise for the induction of

Table 1. Mechanisms of transplantation tolerance

Clonal deletion
Central (thymic)
Peripheral (passive cell death or activation induced cell death)
Microchimerism (possible role of vein cells)
Clonal anergy
Regulatory cells (infectious tolerance)

transplantation tolerance in humans (Table 2), T-cell costimulatory blockade is perhaps the most promising and will likely be tested clinically in the near future. Ligation of CD28 by B7-1 or B7-2 is blocked by CTLA4Ig, a recombinant fusion protein that contains the extracellular domain of soluble CTLA4 fused to an Ig heavy chain (79). CTLA4Ig binds to both B7-1 and B7-2 and acts as a competitive inhibitor of CD28 binding to B7-1 and B7-2, resulting in T cell anergy *in vitro* (80, 81) (see Fig. 3). T cell costimulatory blockade by systemic administration of CTLA4Ig has been very effective in preventing experimental acute rejection, prolonging graft survival and inducing specific tolerance in some transplantation models (10). Recent studies from our laboratory have demonstrated that CD28-B7 T cell costimulatory blockade by CTLA4Ig prevents development of chronic rejection in rat models of cardiac (82) as well as renal (83) transplantation.

More interestingly, CD28-B7 blockade late after acute injury interrupts progression of chronic rejection in an experimental chronic renal allograft rejection model (84). The exact mechanisms mediating induction of tolerance by CD28-B7 T cell costimulatory blockade *in vivo* remain unclear (10). It has been suggested that CTLA4Ig induces T cell anergy and prevents expansion of antigen-specific T cells *in vivo* (26). In the mouse heart transplant model, the induction of unresponsiveness requires an intact CTLA4 negative signaling pathway (85). Our studies in the rat acute renal allograft rejection model indicate that systemic tolerance induced by the administration of CTLA4Ig is associated with selective inhibition of Th1 and sparing of Th2 cytokines in the target organ (51). However, as indicated earlier, recent data in cytokine gene knockout animals highlight the complexity of studying the role of cytokines in allograft rejection and tolerance. To further complicate the picture, inducible costimulator (ICOS), a proposed third member of T cell specific cell surface receptors, CD28 and CTLA4, has recently been identified (86) (see Fig. 1) What is interesting is that unlike constitutively expressed CD28, ICOS must be induced *de novo* on the surface of the T cell and does not upregulate the production of IL-2, but superinduces the synthesis of IL-10. However, the role of ICOS in graft rejection and/or tolerance remains to be determined.

Recently, there has been great interest in studying the role of CD40 and its ligand, CD40L, in the process of allograft rejection and tolerance (10) (see Fig. 4). CD40, a member of the TNF receptor family, is expressed on B cells and other APCs, including dendritic cells and endothelial cells (87). CD40L (a member of the TNF family, also known as gp39), is expressed early on activated T cells (87). Binding of CD40L to CD40 is critical in providing T-cell help for B-cell Ig production and class switching (88); a defect in CD40L is responsible for the hyper-IgM syndrome in humans (89).

The role of CD40L in T-cell activation has been uncertain. Studies using CD40L knockout mice have demonstrated an inability of CD40L-deficient T cells to undergo effective clonal expansion (90, 91). It has been questioned, however, whether CD40L acts directly to transduce a costimulatory signal to the T cell or indirectly, since ligation of CD40 on APCs is a strong inducer of B7 expression (92, 93). Therefore, CD40L on the T cell might merely serve to induce CD28-ligands or other costimulatory molecules on APCs. Several studies in

Table 2. Novel experimental approaches

Intrathymic tolerance
Bone marrow tolerance
-augmentation and reconstitution
Class I and class II
MHC-derived peptides and MHC proteins
Costimulatory T cell activation pathway blockade:
CTLA4Ig, anti-B7 antibodies and humanized anti-CD40L
Gene targeted therapies:
Fas, transcription, cytokines and immunomodulatory agents

Table 3. Transplantation tolerance: why is it difficult to translate animal studies to humans

Dependence on genetically defined inbred strains of small animals
Pathogen-free small animal facilities
Differences in expression of class II MHC and/or costimulatory molecules on endothelial cells and T cells between species
Involution of adult human thymus
Effect of conventional immunosuppression

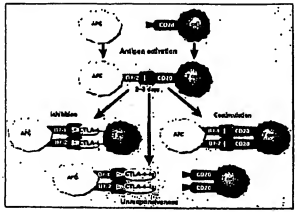

Fig. 3. Functions of CD28, B7-1, B7-2 and CTLA-4 molecules. Resting T cells express CD28, but concurrently resting APCs do not express B7 molecules. Within six h of activation, B7-2 is expressed by APCs and becomes available for binding to CD28, transmitting a costimulatory signal to the T cell. By 48–72 h after activation, APCs also express B7-1, while T cells express the CTLA-4 inhibitory receptor. Both B7-1 and B7-2 may bind either to CD28 or CTLA-4, providing continued costimulation or a new inhibitory signal, respectively. Since CTLA-4 has a higher affinity for B7 molecules than CD28, its inhibitory interaction ultimately prevails, leading to immune response termination. The fusion protein CTLA4Ig may compete with CD28 and CTLA-4 for B7 binding, thus blocking costimulatory interactions. [Reprinted with permission: N Engl J Med 1998;338:1813–21. © 1998 Massachusetts Medical Society. All rights reserved.]

experimental transplantation models indicate that CD40L blockade can prevent acute rejection and prolong allograft survival (52, 94, 95). Larsen et al. (55) addressed the potential for synergy between B7 and CD40L blockade by demonstrating that inhibition of these two pathways leads to prolonged allogeneic mouse skin and cardiac allograft survival. An interesting finding in this study was that only combination therapy with both anti-CD40L and CTLA4Ig was able to prevent graft arteriosclerosis and fibrosis (chronic rejection). Recent studies in islet (96) as well as renal (97) primate transplant recipients indicate the efficacy of T cell costimulatory approaches in preclinical models and provide the rationale to develop such strategies in human organ transplant recipients.

One of the interesting observations in several of the experimental studies with T cell costimulatory blockade is the fact that administration of donor antigen appears to synergize with CTLA4Ig or anti-CD40L in promoting long-term graft survival (52, 95, 98, 99). In some models, the administration of donor cells was necessary to prevent the development of chronic rejection (56, 100). More interestingly, a recent study by Wekerle et al. showed that induction of chimerism with donor bone marrow and T cell costimulatory blockade results in delictional tolerance. This strategy may have applicability

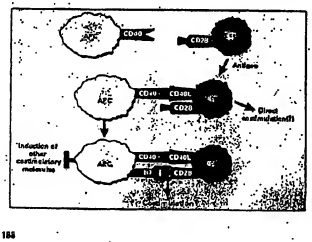

Fig. 4. The CD40L–CD40 pathway and its association with the CD28–B7 pathway. Resting APCs, that include B cells, macrophages and dendritic cells express CD40. When activated, T cells express CD40 ligand. The CD40L–CD40 interaction is important in providing T cell help to prevent apoptosis as well as to induce immunoglobulin production and isotype switching. Activation of CD40 on APCs provides a signal for B7 induction, especially B7-2. CD40 ligand may act in T cell costimulation by directly providing costimulation, by inducing B7, or by inducing other costimulatory ligands. [Reprinted with permission: N Engl J Med 1998;338:1813–21. © 1998 Massachusetts Medical Society. All rights reserved.]

to humans, because it may allow clinical translation of the bone marrow chimerism approach, without myeloablation or T cell depletion of the host (19).

After a decade of laboratory studies, CTLA4Ig has now been used in the clinical arena. Abrams and others have recently published the results of a phase I clinical trial describing the immunosuppressive effects of CTLA4Ig in the T cell mediated autoimmune disease, psoriasis vulgaris (101). This timely initial trial serves to underscore the possible application of costimulatory blockade to various clinical diseases, including transplant rejection (102).

Clinical tolerance

Despite the fact that it has been relatively easy to induce true tolerance in small experimental animals, translating these studies into larger animals and humans has been much more difficult to achieve. Some of the hurdles that may explain this dilemma are summarized in Table 3. Even if we have the ideal strategy to use in humans, the lack of reliable predictive assays for rejection or tolerance still does not allow us to know if a patient is truly tolerant so that immunosuppressive agents may be withdrawn. Rechallenging a transplant recipient with a second test graft to prove tolerance is not feasible. Therefore, we must define achievement of transplantation tolerance in clinical, immunologic and molecular terms. Once we accomplish this task, transplantation tolerance will no longer be an elusive goal.

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blockade of the CD40-gp39 pathway. Transplantation 1996: 61: 4–9.

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97. Kirk AD, Harlan DM, Armstrong NN, et al. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. Proc Natl Acad Sci USA 1997: 94: 8789–8794.

98. Lin H, Bolling SF, Linsley PS, et al. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. J Exp Med 1993: 178: 1801–1806.

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102. Sayegh M. CTLA4Ig finally graduates to the clinic. J Clin Invest 1999: 103: 1223.

EXHIBIT I

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Habib Zaghouani, et al.	Title:	Sustained Treatment of Type 1 Diabetes After Expression of Predisposition Markers
App. No.:	10/681,788		
Conf. No.:	6701	Art Unit:	1644
Filing Date:	October 8, 2003	Examiner:	Edwoldt, Gerald R.

Mail Stop: Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

I, Habib Zaghouani, do hereby declare and say:

1. I am a citizen of the United States and my current residential address is 1608 Brookfield Manor, Columbia, Missouri, 65203.
2. I obtained my undergraduate degree in biochemistry from University of Paris, France in 1981. I obtained a Ph.D. in immunology from the University of Paris/Cancer Research Institute, France in 1987.
3. I am presently the J. Lavenia Edwards Chair in Pediatrics, Director, Center for Cellular and Molecular Immunology and Professor, Department of Molecular Microbiology & Immunology and Department of Child Health at the University of Missouri.
4. I have over one hundred publications and abstracts in the field of immunology. Please refer to the copy of my *curriculum vitae* in attached Appendix A for more details.
5. I am a named inventor on the '788 application as well as on related co-pending application serial numbers: 10/510,411; 11/290,070; and 11/425,084.
6. I have performed an experiment examining the impact of administration, initiated at the pre-diabetic stage, of soluble Ig-GAD2 to NOD mice over a period of 56 weeks. Data are provided in attached Appendix B.
7. NOD mice were assessed for blood glucose beginning at week 12 of age. Those mice that reached glucose levels of 160 – 250 mg/dl between week 14 to 25 received the following Ig-GAD2 regimen: 500 μg of soluble Ig-GAD2 i.p. daily for 5 days and then weekly injections thereafter for either 15 or 25 weeks. Blood glucose monitoring was performed during this period.
8. Overall, 100% of mice that became pre-diabetic at the age of 14 – 25 weeks and that were not treated with Ig-GAD2 progressed to diabetes (blood sugar level 300 mg/dl glucose) within 5 weeks after diagnosis of the pre-diabetic stage. Moreover, 60% of mice undergoing the 15-week treatment regimen were protected against diabetes throughout the

25 week post-hyperglycemia monitoring period. Interestingly, one mouse (Figure 1 B, left panel, open stars) progressed to diabetes by 5 weeks of treatment and 3 mice (Figure 1 B, plus, open diamond, and open pentagon) had similar disease manifestations shortly after interruption of the treatment.

9. When the regimen was extended to 25 weeks, 100% of the Ig-GAD2 treated animals were protected (Figure 1 A, right panel) and normoglycemia was restored in all mice (Figure 1 B, right panel). This status persisted throughout the duration of the study, which was terminated when the mice were 52 to 56 weeks of age.
10. Blood glucose levels for sol Ig-GAD2 (through week 24 of treatment) treated mice are shown in Table 1. Blood glucose levels for the untreated mice are shown in Table 2.
11. It is my professional opinion that the NOD mouse model is an appropriate and well accepted animal model for Type I diabetes.
12. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

December 19, 2007

Date

Habib Zaghouani, PhD

APPENDIX A

Curriculum Vitae Habib Zaghouani

EDUCATION

Ph.D.	1987	Immunology, University of Paris/Cancer Research Institute, Paris, France.
M.S.	1983	Immunology, University of Paris/Pasteur Institute, Paris, France.
B.S.	1981	Biochemistry, University of Paris, Paris, France.

POSITIONS AND RESEARCH EXPERIENCE

2006-present	Director, Center for Cellular and Molecular Immunology, The University of Missouri School of Medicine, Columbia, MO
2006-present:	J. Lavenia Edwards Chair in Pediatrics, the University of Missouri School of Medicine, Columbia, MO
2006-present:	Professor, Department of Child Health, the University of Missouri School of Medicine, Columbia, MO.
2001-present:	Professor, Department of Molecular Microbiology and Immunology, the University of Missouri School of Medicine, Columbia, MO.
2000-2001:	Associate Professor, Department of Microbiology, the University of Tennessee, Knoxville, Tennessee.
1994-2000:	Assistant Professor, Department of Microbiology, the University of Tennessee, Knoxville, Tennessee.
1990-1994:	Research Assistant Professor, Department of Microbiology, Mount Sinai School of Medicine, New York.
1987-1989:	Postdoctoral Fellow, Department of Microbiology, Mount Sinai School of Medicine, New York. Mentor: Dr. Constantin A. Bona.
1983-1987:	Graduate Research Assistant, Ph.D. candidate, Immunology, University of Paris/Cancer Research Institute, Paris, France. Mentor: Dr. Marc Stanislawski.
1981-1983:	Graduate Research Assistant, M.S. candidate, Immunology, Pasteur Institute, Paris, France. Director: Dr. Arthur Dony Strosberg.

RESEARCH GRANT SUPPORT

A. Principal Investigator

Active

1). **2RO1 NS 037406,** National Institutes of Health, March 2004 - February 2009. Modulation of autoreactive T cells. PI: Habib Zaghouani.

2). **1RO1 DK 065748,** National Institutes of Health, April 2005-March 2008. Immune tolerance against type I diabetes in mice. PI: Habib Zaghouani.

3). **2RO1 AI 48541,** National Institutes of Health, May 2006- April 2011. Regulation of neonatal immunity. PI: Habib Zaghouani.

4). **1R21 AI 068746,** National Institutes of Health. July 2007 – June 2009. Mimotopes against type I diabetes. PI: Habib Zaghouani.

Pending

1). **1RO1 NS057194-A2,** National Institutes of Health, April 2008 - March 2013. Regulation of autoimmune encephalomylitis. PI: Habib Zaghouani.

2). **2RO1 DK 065748-01,** National Institutes of Health, April 2008-March 2013. Immune tolerance against type I diabetes in mice. PI: Habib Zaghouani.

B. Co-investigator, Mentor, or Key Personnel

Active

T32 GM008396, National Institute of General Medical Sciences (NIGMS), July 1991-June 2012. Molecular Basis of Gene Expression and Signal Processing. PI: Mark Hannink (Zaghouani: Mentor).

T32 RR007004, National Institutes of Health, July 2005-June 2010, Postdoctoral Training in Comparative Medicine. PI: Craig Franklin (Zaghouani: Mentor).

T90 DK71510, National Institutes of Health, September 2004 – August 2009. Bench and Back: Clinical biodetectives training. PI: Mark Milanick (Zaghouani: Mentor).

R90 DK71510, National Institutes of Health, September 2004 – August 2009. Bench and Back: Clinical biodetectives training. PI: Mark Milanick (Zaghouani: Mentor).

KO8 AR048671, National Institutes of Health, June 2005-April 2008, Cytokine regulation of collagen-induced arthritis. PI: Robert Ortman (Zaghouani: Mentor).

1G20 RR021327, National Institutes of Health, September 2004-August 2009. Equipment for the MU Life Sciences Center. PI: Lon Dixon, (Zaghouani: Key personnel).

1 G20 RR019711, National Institutes of Health, September 2004-Agust 2009. Renovation of MU Medical School Vivarium. PI: Lon Dixon. (Zaghouani: Key personnel).

U19AT003264-01, National Institutes of Health, September 2005 – August 2009. TICIPS: HIV/AIDS, Secondary Infections and Immune Modulation. Center grant. PI: William Folk (Zaghouani: Faculty Member).

Research Foundation Grant, Arthritis Foundation, April 2006 – May 2008. Synoviolin is a target for arthritis. PI: Deyu Fang (Zaghouani: Mentor).

C. Previous Support (PI: Zaghouani, H)

1). R21 AI 062796, National Institutes of Health, July 2005-June 2007. Immune tolerance in the newborn mouse. Yearly direct cost $150,000. PI: Habib Zaghouani. No cost extension 11/30/2007

2). 1RO1 AI48541, National Institutes of Health, May 2001- April 2006. Regulation of neonatal immunity. Yearly direct cost: $175,000. PI: Habib Zaghouani.

3). Astral Inc, October 2001- September 2004. Development of Approaches to Combat Autoimmunity. PI: Habib Zaghouani.

4). RO1NS37406, National Institutes of Health, January 2000- December 2004. Modulation of autoreactive T cells. PI: Habib Zaghouani

5). RG2967B-3, National Multiple Sclerosis Society, October 2002 – March 31, 2004 Down-regulation of encephalitogenic T cells. PI: Habib Zaghouani.

6). RG2967A2/1, National Multiple Sclerosis Society, April 99 - March 2002. Down-regulation of encephalitogenic T cells. PI: Habib Zaghouani.

7). Astral Inc: March 95 - July 2001. A novel approach to delete encephalitogenic T cells. PI: Habib Zaghouani.

8). RG2778A1/1, National Multiple Sclerosis, April 96 - March 1999. A deletional strategy for encephalitogenic T cells. PI: Habib Zaghouani.

9). Astral Inc:, September 97- August 99. Generation of human Ig chimeras carrying wild type or antagonist forms of myelin peptides. PI: Habib Zaghouani.

10). 1R41AI47496, (STTR): National Institutes of Health, September 2000-August 2001. Treatment of EAE using a novel delivery system. . Co-PI: Habib Zaghouani.

TEACHING EXPERIENCE

2004:	Microbiology 205 (Medical Microbiology) 3 credit hours, 8 lecture contact hours, 170 student, Spring semester, University of Missouri School of Medicine, Columbia.
2002-present:	Microbiology 304 (Immunology) 3 credit hours, 14 lecture contact hours, 30 students, Fall semester, Molecular Microbiology and Immunology, University of Missouri School of Medicine, Columbia.
2002-present	Microbiology 407 (advanced Immunology) 4 credit hours, 9 lecture contact hours, 18 students, Spring semester, Molecular Microbiology and Immunology, University of Missouri School of Medicine, Columbia.
2001-present:	Bio 4952, Undergraduate research, 3 credit hours, 1-2 students, Fall and Winter semesters
2001-present:	Bio 4950, Undergraduate research, 3 credit hours, 2-3 Students, Fall and Winter semesters
2001-present:	Direct Immunology Journal Club, 1hour/week all year around, 40 student, postdocs and faculty members
1995-2001:	Microbiology 430 (Immunology), 3 credit hours, 45 lecture contact hours, 100-120 students, Fall semester, Microbiology, The University of Tennessee, Knoxville.
1995-2001:	Co-direct Microbiology 602 (Microbial Pathogenesis Journal Club), 1 credit hour, 15 lecture contact hours, 10-15 students, Fall semester, Microbiology, The University of Tennessee, Knoxville.
1995-2001:	Co-direct Microbiology 603 (Immunology Journal Club), 1 credit hours, 15 lecture contact hours, 10-15 students, Spring semester, Microbiology, The University of Tennessee, Knoxville.
1995-2001:	Microbiology 401 (Undergraduate Research), 3 credit hours, 1-2 students per semester, Microbiology, The University of Tennessee, Knoxville.

1998: Microbiology 630 (Topics in Immunology), 3 credit hours, 10 lecture contact hours, 20 students, Spring semester, (Seminar Series) Microbiology, The University of Tennessee, Knoxville.

1998-2001: Microbiology 493 (Independent Study in Immunology), 6 students, 10 lecture contact hours, spring, Microbiology, The University of Tennessee, Knoxville.

1992-1994: 600-level Immunology course, 3 credit hours, 6 lecture contact hours, 10 students, spring, Microbiology, Mount Sinai School of Medicine, New York.

HONORS AND AWARDS

2006. Speaker, Keystone Symposia on Tolerance Autoimmunity and Immune Regulation. March 21-26, 2006. Beaver Run Resort, Breckenridge, Colorado. Presentation title: Tregs for or against diabetes.

2004: Research Equipment Award for the purchase of an ELISPOT Analyzer, Office of Research, The University of Missouri,

2003: Keystone Symposia Scholarship ($1,000) for poster presentation by Hyun-Hee Lee, a graduate student in the laboratory, the meeting was held in Snowbird, UT

2003: Honorable citation for poster presentation by Randal Gregg, a graduate student in the laboratory. Life Science week, University of Missouri-Columbia.

2001: Science Alliance Research Excellence Award, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.

2000: Science Alliance Research Excellence Award, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.

2000: Exhibit, Performance, and Publication Expense Award, Faculty Senate Research Council and Office of research Administration, The University of Tennessee, Knoxville.

1999: Chancellor's nomination for Howard Hughes Medical Institute Assistant Investigator Appointment, The University of Tennessee, Knoxville.

1999: Biological Equipment Award, Office of Research Administration/Science Alliance/Genome Science and Technology/Division of Biology, The University of Tennessee, Knoxville.

1999: Science Alliance Research Excellence Award, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.

1999: Exhibit, Performance, and Publication Expense Award, Faculty Senate Research Council and Office of research Administration, The University of Tennessee, Knoxville.

1998: Science Alliance Research Excellence Award, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.

1998: Exhibit, Performance, and Publication Expense Award, Faculty Senate Research Council and Office of Research Administration, The University of Tennessee, Knoxville.

1997: Biological Equipment Award, Office of Research Administration/Science Alliance/ Division of Biology/ Department of Microbiology, The University of Tennessee, Knoxville.

1997: Exhibit, Performance, and Publication Expense Award, Faculty Senate Research Council and Office of Research Administration, The University of Tennessee, Knoxville.

1990: Research Excellence Award, Alliance Pharmaceutical Corporation. San Diego, CA.

1987-1988: Scientist Exchange Award (Postdoctoral Fellowship), French Cancer Society, Paris, France.

1984-1987: Graduate Student Scholarship, French Cancer Society, Paris, France.

PROFESSIONAL SERVICE

2007: Chair, Block symposium, regulation of immune cell development and function, American Association of Immunologis, Miami, FL.

2006-2010: Panel member, Hypersensitivity, Autoimmune and Immune-mediated Diseases (HAI) study section.

2006: Chair, Block symposium, treatment of autoimmune disease, American Association of Immunologis, Boston, MA.

2006: Review panel member, research proposals on Neurosciences, La Marato de TV3 Foundation, Catalan Agency For Health Technology Assessment And Research

2005: Chair, Block symposium, Cytokines and autoimmunity, American Association of Immunologis, Experimental Biology Meeting, San Diego, CA.

2004:	Panel member, NIAID Biodefence Workshop, Immunization and Vacination in Special Populations, Division of Allergy, Immunology and transplantation, NIH, Bethesda, MD
2004:	Chair, Block symposium, Tolerance and regulation of autoimmunity, American Association of Immunologis, Experimental Biology Meeting, Washington DC.
2004-present:	Adhoc Reviewer, TTT Study section, National Institutes of Health
2004-present:	Adhoc Reviewer, HAI Study section, National Institutes of Health
2003	Adhoc Reviewer, IMS Study Section, National Institutes of Health
2003	Adhoc Reviewer, ALY Study Section, National Institutes of Health
2003-present:	Member, Molecular Biology Program, University of Missouri-Columbia
2003-present:	Member, Genetics Area Program, University of Missouri-Columbia
2003-present:	Member, Veterinary Pathobiology Area Program, University of Missouri-Columbia
2003-present	Scientific Consultant, Division of endocrinology and Diabetes, Unversity of Missouri, Kansas City, MO
2002-2004:	Scientific Consultant, Alliance Pharmaceutical, San Diego, CA.
2001-present:	Member of The Graduate Student Recruitment Committee, Department of Molecular Microbiology and Immunology, The University of Missouri School of Medicine, Columbia.
2000-2001:	Adhoc Reviewer, BM-1 Study Section, National Institutes of Health
1992-2000:	Editorial board member: *Viral Immunology*
1989-present:	Reviewer: Immunology Journals
2000:	Guest Editor, International Review of Immunology
2000-2001:	Chair, Graduate Student Advisory Committee, Genome, Science, and Technology program, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.
1995-2001:	Member of The Graduate Student Recruitment Committee, Department of Microbiology, The University of Tennessee, Knoxville.
1998:	Member of Faculty Search Committee, Department of Comparative Medicine, College of Veterinary Medicine, The University of Tennesee, Knoxville.

1999: Panel Member: NIH/NCI, Small Business Innovation Research (SBIR)/Small Business Technology Transfer (STTR) Grant program. Flexible system to advance innovative research for cancer drug discovery by small business panel.

PROFESSIONAL MEMBERSHIP

2006-present: Member of the Henry Kunkel Society
1998-present: Member of the Society for Neuroscience
1992-present: Member of the American Association for the Advancement of Science.
1992-present: Member of the American Association of Immunologists.

PUBLICATIONS

Manuscripts published in peer-review journals

59. Bot, A., D. Smith, B. Phillips, S. Bot, C. Bona, and H. Zaghouani. **(2006).** Immunologic control of tumors by *in vivo* FcgR-targeted antigen loading in conjunction with dsRNA-mediated immune modulation. *J. Immunol.* 176:1363-1374.

58. Caprio-Young, J., J. J. Bell, H-H. Lee, J. S. Ellis, D. M. Nast, G. Sayler, B. Min, and H. Zaghouani. **(2006).** Neonatally Primed Lymph Node but not Splenic T Cells Display a Gly- Gly Motif Within the T Cell Receptor Beta Chain Complementarity Determining Region 3 (CDR3) That Controls Affinity and Lymphoid Organ Retention. *J. Immunol.* 176:357-364.

57. Yu, P., R. K. Gregg, J. J. Bell, J. S. Ellis, R. Divekar, H-H Lee, R. Jain, H. Waldner, J. C. Hardaway, M. Collins, V. K. Kuchroo, and H. Zaghouani. **(2005).** Specific T regulatory cells (Tregs) display broad suppressive functions against experimental allergic encephalomyelitis upon activation with cognate antigen. *J. Immunol.* 174:6772-6780.

56. Gregg, R. K., J. J. Bell, H-H. Lee, R. Jain, S. J. Schoenleber, R. Divekar, and H. Zaghouani **(2005).** IL-10 diminishes CTLA-4 expression on islet-resident T cells and sustains their activation rather than tolerance. *J. Immunol.* 174: 662-670.

55. Gregg, R. K., R. Jain, S. J. Schoenleber, R. Divekar, J. J. Bell, H-H. Lee, P. Yu, and H. Zaghouani. **(2004).** A sudden decline in active membrane-bound TGFβ impairs both T regulatory cell function and protection against autoimmune diabetes . *J. Immunol.* 173:7308-7316.

54. Li, L, H-H. Lee, J. J. Bell, R. K. Gregg, J. S. Ellis, A. Gessner, and H. Zaghouani. **(2004).** IL-4 Utilizes an Alternative Receptor to Drive Apoptosis of Th1 Cells and Skews Neonatal Immunity Towards Th2. *Immunity.* 20: 429-440.

53. Bell, J. J., B. Min, R. Gregg, H-H. Lee, and H. Zaghouani. **(2003).** Break of neonatal Th1 tolerance and exacerbation of experimental allergic encephalomyelitis by interference with B7 costimulation. *J. Immunol.* 171:1801-1808.

52. Legge, K. L., Gregg, R. K. Maldonado-Lopez, R., Li, L., Caprio, J. C., Moser, M., and Zaghouani, H. **(2002).** On the role of dendritic cells in peripheral T cell tolerance and modulation of autoimmunity. *J. Exp. Med.* 196:217-227.

51 Pack, C. D., Cestra, A. E., Min, B., Legge, K. L., Li, L., Caprio, J. C., Bell, J. J., Gregg, R. K., and Zaghouani, H. **(2001).** Neonatal exposure to antigen primes the immune system to develop responses in various lymphoid organs and promotes bystander regulation of diverse T cell specificities. *J. Immunol.* 167:4187-4195

50. Li, L., Legge, K. L, Min, B., Bell, J. J, Gregg, R., Caprio, J. and Zaghouani, H. **(2001).** Neonatal immunity develops in a transgenic TCR transfer model and reveals a requirement for elevated cell input to achieve organ-specific responses. *J. Immunol.* 167:2585-2594

49. Min, B., Legge, K. L., Li, L., Caprio, J. C., Gregg, R. K., Bell, J. J., and Zaghouani, H. **(2001).** Defective expression of CD40L undermines both IL-12 production by antigen presenting cells and up-regulation of IL-2 receptor on splenic T cells and perpetuates INFγ-dependent T cell anergy. *J. Immunol.* 166:5594-5603

48. Day, R. B., Okada, M., Ito, Y., Tsukada, K., Zaghouani, H., Shibuya, N., and Stacey, G. **(2001).** Binding site of chitin oligosaccharides in the soybean plasma membrane. *Plant. Phys.* 126:1-12.

47. Legge, K. L., Min, B., Caprio, J. C., Li, L., Gregg, R. K., Bell, J. J., and Zaghouani, H. **(2000).** Coupling of peripheral tolerance to endogenous IL-10 promotes effective modulation of myelin-activated T cells and ameliorates experimental allergic encephalomyelitis. *J.Exp.Med.* 191:2039-51.

46. Anderson, A. C., Nicholson, L. B., Legge, K. L., Turchin, V., Zaghouani, H., and Kuchroo, V. K. **(2000).** High frequency of auto-reactive myelin proteolipid protein (PLP)-specific T cells in the periphery of naïve mice: mechanisms of selection of the self-reactive repertiore. *J. Exp. Med.* 191:761-770.

a)

45. Min, B., Legge, K. L., Caprio, J. C., Li, L., Gregg, R., and Zaghouani, H. **(2000).** Differential control of neonatal tolerance by antigen dose versus extended exposure and adjuvant. *Cell. Immunol.* 200 :45-55.

44. Legge, K. L., Min, B., Pack, C. D., Caprio, J. C., and Zaghouani, H. **(1999).** Differential

presentation of an altered peptide within fetal central and peripheral organs supports an avidity model for thymic T cell development and implies a peripheral re-adjustment for activation. *J. Immunol.* 162:5738-46.

43. Min, B., Legge, K. L., Pack, C. D. and Zaghouani, H. **(1998).** Neonatal exposure to a self peptide-Ig chimera circumvents the use of adjuvant and confers resistance to autoimmune disease by a novel mechanism involving IL-4 lymph node deviation and INFγ-mediated splenic anergy. *J. Exp. Med.* 188:2007-17.

42. Legge, K. L., Min, B., Cestra, A.E., Pack, C. D., and Zaghouani, H. **(1998).** T cell receptor agonist and antagonist exert in vivo cross-regulation when presented on immunoglobulins. J. Immunol. 161:106-11.

41. Legge, K. L., Min, B., Potter, N.T., and Zaghouani, H. **(1997).** Presentation of a T cell receptor antagonist peptide by immunoglobulins ablates activation of T cells by a synthetic peptide or protein requiring endocytic processing. *J. Exp. Med.* 185:1043-53.

40. Brumeanu, T-D, Dehazya, P., Wolf, I., Bot, A., Bona, C., and Zaghouani, H. **(1996).** Engineering of double antigenized Igs carrying B and T cell epitopes. Immunotechnology 2:85-95.

39. Brumeanu, T-D., Zaghouani, H., and Bona, C. **(1995).** Purification of antigenized immunoglobulins derivatized with monomethoxypolyethylene glycol. *J. Chromatogr.* 696:219-25.

38. Brumeanu, T-D., Zaghouani, H., Elahi, I., Daian, C. and Bona, C. **(1995).** Derivatization with monomethoxypolyethylene glycol of Igs expressing viral epitopes obviates adjuvant requirement. ***J. Immunol.*** 154:3088-95.

37. Zaghouani, H., Anderson, S., Sperber, K. E., Daian, C., Kennedy, R. C., Mayer, L. and Bona, C. **(1995).** Induction of antibodies to the human immunodeficiency virus type 1 by immunization of baboons with immunoglobulin molecules carrying the principal neutralizing determinant of the envelope protein. Proc. Natl. Acad. Sci.USA. 92:631-35.

36. Bona, C., Brumeanu, T-D and Zaghouani, H. **(1994).** Immunogenicity of microbial peptides grafted in self immunoglobulin molecules. Cell. Mol. Biol. 40 (suppl):21-30.

35. Brumeanu, T-D., Swiggard, W. J., Steinman, R. M., Bona, C., and Zaghouani, H. **(1993)**. Efficient loading of identical peptide onto class II molecules by antigenized immunoglobulin and PR8 virus. J. Exp. Med. 178:1795-99.

34. Brumeanu, T-D., Kohanski, R., Bona, C., and Zaghouani, H. **(1993).** A sensitive method to detect defined peptide among those eluted from murine MHC class II molecules. J. Immunol. Meth. 160:65-71.

33. Kuzu, Y., Kuzu, H., Zaghouani, H., and Bona, C. **(1993).** Priming of CTLs at various stages of ontogeny with transfectoma cells expressing a chimeric Ig heavy chain gene bearing an influenza virus nucleoprotein. *International. Immunol.* 5:1301-07.

32. Zaghouani, H., Kuzu, Y., Kuzu, H., Swigard, W., Steinman, R., and Bona, C. **(1993).** Contrasting efficacy of presentation by major histocompatibility complex class I and class II products when peptides are administered within a common protein carrier, self immunoglobulin. *Eur. J. Immunol.* 23:2746-50.

31. Penney, C. L., Ethier, D., Dionne, G., Nixon-George, A., Zaghouani, H., Michon, F., Jennings, H., and Bona, C. **(1993).** Further studies on the adjuvanticity of stearyl Tyrosine and ester analogues. Vaccine. 11:1129-1134.

30. Kuzu, H., Kuzu, Y., Zaghouani, H., and Bona, C. **(1993).** In-vivo priming effect during various stages of ontogeny of an influenza virus nucleoprotein derived peptide. Eur. J. Immunol. 23:1397-1400.

29. Zaghouani, H., Steinman, R., Nonacs, R.,. Shah, H., Gerhard, W. and Bona, C. **(1993)**. Efficient presentation of a viral T helper epitope expressed in the CDR3 region of a self immunoglobulin molecule. *Science*. 259:224-27.

28. Shengqiang, L., Polonis, V., Isobe, H., Zaghouani, H., Guinea, R., Moran, T., Bona, C., and Palese, P. **(1993).** Chimeric influenza virus induces neutralizing antibodies and cytotoxic T cells against human immunodeficiency virus type 1. *J. Virol.* 67:6659-66.

27. Hall, B., Zaghouani, H., Daian, C. and Bona, C. **(1992)**. A single amino acid mutation in CDR3 of the 3-14-9 light chain abolished expression of the IDA 10 defined idiotype and antigen binding. *J. Immunol.* 149:1605-12

26. Nixon, A., Zaghouani, H., Penney, C. L., Lacroix, M., Dionne, G., Anderson, S., Kennedy, R. C. and Bona, C. A. **(1992)**. Adjuvanticity of stearyl tyrosine on the antibody response to peptide 503-535 from HIV gp160. *Viral. Immunol*. 5:141-50

25. Zaghouani, H., Krystal, M., Kuzu, H., Moran, T., Shah, H., Kuzu, Y., Schulman, J. and Bona, C. **(1992)**. Cells expressing a heavy chain immunoglobulin gene carrying a viral T cell epitope are lysed by specific cytolytic T cells. ***J. Immunol.*** 148:3604-09.

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APPENDIX B

Table 1. Blood Glucose Levels (mg/dl) for Treated Mice

	Mouse													
Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	161	165	180	182	165	250	179	212	160	229	232	224	180	173
1	252	125	134	159	144	137	129	179	152	258	183	154	165	199
2	176	119	213	127	144	136	169	165	146	162	227	192	152	148
3	121	117	145	121	117	255	142	228	281	285	217	186	112	111
4	149	110	127	148	126	179	124	151	390	176	214	156	159	132
5	131	144	175	117	116	153	126	128	351	98	99	161	140	136
6	148	132	114	136	150	126	123	143		94	134	97	118	156
7	98	99	146	133	93	105	121	156		89	120	179	135	134
8	128	111	178	152	113	158	119	139		170	127	172	134	108
9	109	104	140	110	120	138	153	147		147	134	142	116	132
10	118	108	160	138	120	140	121	141		145	170	132	117	151
11	151	91	192	144	101	145	113	152		143	112	114	135	163
12	107	91	244	161	130	151	109	216		150	124	149	121	97
13	107	101	256	124	113	137	108	184		142	114	130	148	137
14	85	81	264	125	116	112	119	155		154	127	154	118	143
15	133	113	198	96	120	118	99	156		147	119	178	123	158
16	136	91	285	112	128	103	112	127		153		144	146	157
17	111	129	377	105	111	148	107	134		228		123	123	111
18	127	99	366		98	158	113	148		350		159	122	93
19	99	110			111	176	137	338		339		170	132	
20	94	82			119	152	130	229				256	172	
21	83	96			114	135	153	331					215	
22	75	101				140	133	440						
23	70	99				140	154							
24	90	100				150	118							

Table 2. Blood Glucose Levels (mg/dl) for Untreated Mice

	Mouse						
Week	11	12	13	14	15	16	17
0	174	169	169	168	175	199	219
1	293	366	155	159	251	240	379
2	352	400	157	200	340	450	400
3	457	-	200	249	450	-	-
4	-	-	270	393	-	-	-
5	-	-	376	400	-	-	-
6	-	-	400	-	-	-	-

Figure 1

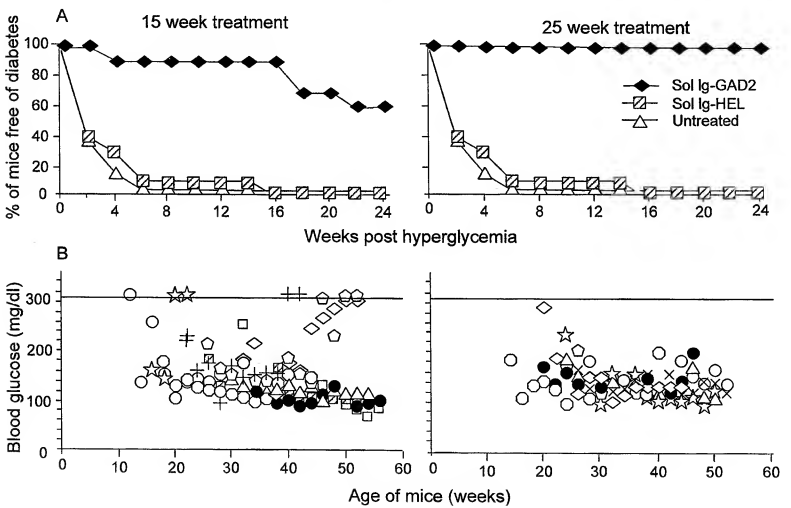
A
15 week treatment
25 week treatment
% of mice free of diabetes
100
80
60
40
20
0
0
4
8
12
16
20
24
Sol Ig-GAD2
Sol Ig-HEL
Untreated
Weeks post hyperglycemia
B
Blood glucose (mg/dl)
300
200
100
0
0
10
20
30
40
50
60
Age of mice (weeks)

EXHIBIT J

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Habib Zaghouani
Appl. No.:	10/681,788
Conf. No.:	6701
Filed:	October 8, 2003
Title:	SUSTAINED TREATMENT OF TYPE 1 DIABETES AFTER EXPRESSION OF PREDISPOSITION MARKERS
Art Unit:	1644
Examiner:	Edwoldt, G.R.
Docket No.:	0119742-005

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CONTINUED EXAMINATION AND RESPONSE TO OFFICE ACTION DATED MARCH 28, 2008

Sir:

This paper is submitted in response to the Office Action dated March 28, 2008.

A **Petition for Extension of Time** appears on page 2.

A **Request for Continued Examination** begins on page 3.

An **Amended Abstract** is provided on page 4.

Amendments to the Claims are reflected in the listing of claims which begins on page 5.

Remarks/Arguments begin on page 9.

A **Conclusion** is provide on page 21.

ABSTRACT

The present invention relates generally to ~~the use of compounds, compositions, combinations, kits, and~~ methods and compositions for treatment, prevention, suppression, and/or delaying the onset of type 1 diabetes. More specifically, the present invention relates to the administration of a fusion protein comprising at least one immunoglobulin having one or more diabetogenic epitopes inserted within the variable region, for suspending, preventing or delaying the onset of type 1 diabetes.

IN THE CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application.

Complete Listing of Claims:

1. (Currently Amended) A method of ~~suspending,~~ preventing or delaying onset of type 1 diabetes in a subject in need thereof, the method comprising administering to the subject a pharmaceutically acceptable composition comprising a soluble fusion protein, wherein the fusion protein comprises at least one immunoglobulin having a variable region comprising a CDR1, a CDR2, or a CDR3 region, the at least one immunoglobulin having at least one protein fragment or peptide inserted within the variable region; wherein (a) the protein fragment or peptide is ~~selected from the group consisting of a protein fragment or peptide derived from INS, a protein fragment or peptide derived from GAD where said protein fragment or peptide derived from GAD comprises GAD1 or~~ GAD2 represented by SEQ. ID NO 4, ~~a diabetogenic epitope, and a T cell receptor engaging determinant, and~~ (b) the subject has undergone insulin autoantibody seroconversion prior to said administering step and (c) the composition is administered to the subject in one or more dosage administrations.

2. (Original) The method of claim 1, wherein the immunoglobulin is human or humanized.

3. (Currently Amended) The method of claim 1, wherein the subject is a human subject ~~that has undergone IAA seroconversion~~.

4. (Previously presented) The method of claim 1, wherein administration of the composition to the subject results in down regulation of an autoreactive T cell.

5. (Previously presented) The method of claim 1, wherein the at least one protein fragment or peptide is inserted within a CDR region of the at least one immunoglobulin.

6. (Cancelled)

7. (Previously presented) The method of claim 5, wherein administration of the composition to the subject results in substantially reduced activation of an autoreactive T cell specific for the at least one protein fragment or peptide.

8. (Withdrawn) The method of claim 1, wherein the at least one protein fragment or peptide is derived from INS.

9. (Withdrawn) The method of claim 8, wherein the INS comprises soluble INSß.

10. (Withdrawn) The method of claim 9, wherein the soluble INSß is capable of binding to at least one Fc receptor.

11. (Withdrawn) The method of claim 10, wherein the Fc receptor is a Fcγ receptor.

12. (Withdrawn) The method of claim 10, wherein the composition is capable of being endocytosed by antigen presenting cells.

13. (Currently amended) The method of claim 1, wherein the at least one protein fragment or peptide ~~is derived from GAD65 and comprises GAD1 or~~ consists essentially of GAD2 represented by SEQ. ID NO 4.

14. (Cancelled)

15. (Previously presented) The method of claim 13, wherein the subject is GAD positive.

16. (Previously presented) The method of claim 1, wherein the subject has not developed hyperglycemia at initiation of the administering step.

17. (Previously presented) The method of claim 1, wherein the subject expresses a type 1 diabetes predisposition marker at initiation of the administering step.

18. (Previously presented) The method of claim 1, wherein upon administration of the composition to the subject, the subject undergoes a dose dependent suspension, prevention, or delay in onset of type 1 diabetes.

19. (Previously presented) The method of claim 1, wherein administration of a first dosage of the composition occurs before the subject has developed type-1 diabetes.

20. (Withdrawn) A composition for suppressing the onset of type 1 diabetes in a subject that has undergone IAA seroconversion, the composition comprises: a pharmaceutically acceptable composition comprising at least one immunoglobulin selected from the group consisting of INS, GAD, an insulin protein, a peptide derived from insulin, a diabetogenic epitope, and a T cell receptor engaging determinant.

21. (Withdrawn) The method of claim 20 wherein the fusion protein is in soluble form.

22. (Previously presented) The method of claim 2 wherein the immunoglobulin is selected from the group consisting of IgG1, IgG2, IgG2a, IgG2b, IgG3, IgG4, IgGA, IgA1, IgA2, IgGE, IgD, IgE, or IgM.

23. (Previously presented) The method of claim 5 wherein the at least one protein fragment or peptide is inserted within the CDR3 region of the immunoglobulin.

24. (Previously presented) The method of claim 23 wherein the at least one protein fragment or peptide is inserted within the CDR3 region of the immunoglobulin in place of a D segment.

25. (Withdrawn) The method of claim 13 wherein the at least one protein fragment or

peptide consists of amino acid residues 524-543 of GAD65.

26. (Currently amended) The method of claim 13 wherein the at least one protein fragment or peptide ~~derived from GAD65 comprises~~ consists of amino acid residues 206-220 of GAD65.

27. (New) The method of claim 13 wherein the subject is a human.

28. (New) The method of claim 1 wherein the pharmaceutical composition further comprises at least one pharmaceutically acceptable carrier.

29. (New) The method of claim 28 wherein the composition comprises an aqueous solution or suspension.

30. (New) The method of claim 29 where the administering step is accomplished by injection or infusion.

REMARKS/ARGUMENTS

By the present proposed amendment, four (4) claims are amended, zero (0) claims are cancelled, and four (4) new claims are added. No fees for claims are believed payable.

Support for amended claim 1 can be found in the specification as filed at least on page 13, line 6, page 21, line 10, and page 45, line 25 – page 46, line 1.

Support for amended claim 13 can be found in the specification as filed at least on page 45, line 25 – page 46, line 1.

Support for new claim 27 can be found in the specification as filed on page 12, line 13.

Support for new claims 28 – 30 can be found in the specification as filed at least on page 13, lines 15 – 24.

No new matter has been added by the present proposed claim amendments and no change in inventorship is believed to result. Entry of the proposed amendments is respectfully requested.

I. Rejection Under 35 U.S.C. § 112, first paragraph – Written Description Rejection.

Claims 1-5, 7, 13, 15-19 and 22-24 stand rejected under 35 U.S.C. § 112, first paragraph as allegedly containing subject matter which was not described in the specification in such a way to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. Applicants respectfully traverse this rejection.

Applicants propose to amend the claims solely to expedite prosecution of certain embodiments of the present invention. Specifically, Applicants' proposed claim amendments make clear that "the protein fragment or peptide is GAD2 represented by SEQ. ID NO. 4" which, as is clearly defined in the specification, corresponds to amino acids 206-220 of GAD 65. (See at least page 45, line 20 – page 46, line 1 which defines GAD 2 as SEQ. ID NO. 4 corresponding to amino acids 206-220 of GAD 65). Withdrawal of this rejection is therefore respectfully requested.

II. Rejection under 35 U.S.C. § 112, first paragraph – Utility Rejection.

Claims 1-5, 7, 13, 15-19 and 22-26 stand rejected under 35 U.S.C. § 112, first paragraph on the alleged basis that there is "insufficient evidence that the claimed method could effectively

function as a method for suspending, preventing or delaying the onset of type 1 diabetes (IDDM)." OA at 3. Thus, the instant rejection is a utility rejection under 35 U.S.C. § 112, first paragraph grounded on the basis that the asserted utility is credible. Applicants respectfully traverse this rejection.

The MPEP states that "[o]ffice personnel should not impose a 35 U.S.C. 112, first paragraph rejection grounded on a 'lack of utility' basis **unless** a 35 U.S.C. 101 rejection is proper." MPEP 2164.07 IA and MPEP 2107.01 IV. (emphasis added). In particular, the factual showing needed to impose a rejection under 35 U.S.C. 101 must be provided if a 35 U.S.C. 112, first paragraph, rejection is to be imposed on 'lack of utility' grounds. *Id.*

As will be discussed in detail below, since the factual showing needed to impose a rejection under 35 U.S.C. 101 has ***not*** been made, the 35 U.S.C. 112, first paragraph, rejection must also fail.

1. A specific, substantial and credible utility has been established.

Applicants have asserted a specific, substantial and credible utility clearly meeting the utility requirements of 35 U.S.C. 101. Since a 35 U.S.C. 101 rejection is not proper, neither is a 35 U.S.C. 112, first paragraph rejection grounded on 'lack of utility' proper.

A. Specific Utility.

MPEP 2107.I.A provides that "[a] 'specific utility' is specific to the subject matter claimed and can 'provide a well-defined and particular benefit to the public'." Applicants have met this burden by, *inter alia*, asserting a method of preventing or delaying onset of type 1 diabetes in a subject in need thereof. This utility is specific to the subject matter claimed and clearly provides a well-defined and particular benefit to the public—prevention or delay of onset of type 1 diabetes. MPEP 2107.01I.A.

B. Substantial Utility.

MPEP 2107.01.I.B makes clear that to satisfy the "substantial utility" requirement, an asserted use must show that the claimed invention has a significant and presently available benefit to the public. Applicants submit that they have met this burden by, *inter alia*, asserting a method of preventing or delaying type 1 diabetes in a subject in need thereof—a significant, immediate and well-defined public benefit to be sure. "Courts have repeatedly found that the mere identification of a pharmacologic activity of a compound that is relevant to an asserted

pharmacological use provides an 'immediate benefit to the public' and thus satisfies the utility requirement." MPEP 2107.01 III (emphasis in original).

C. Credible Utility.

The Office Action suggests that because the claimed composition has not yet been shown to prevent or delay the onset of diabetes in humans that the utility is not credible. In particular, at page 3, the Office Action states that "[s]pecifically, the specification provides insufficient evidence that the claimed method could effectively function as a method for suspending, preventing or delaying the onset of type 1 diabetes (IDDM)". The Office Action further states that "it appears that the only actual evidence of record supports the position that the results of therapies in animal models cannot be used to predict the results of the same therapies in humans." OA at 6.

As an initial matter, Applicants point out that many of the claims are not limited to treatment of humans. As such, the Examiner's position that success in an animal model does not necessarily translate to success in humans is irrelevant with respect to such claims. Furthermore, the MPEP makes clear that "[c]redibility is assessed from the perspective of one of ordinary skill in the art in view of the disclosure and any other evidence of record (*e.g.*, test data, affidavits or declarations from experts in the art, patents or printed publications) that is probative of the applicant's assertions. An applicant need only provide one credible assertion of specific and substantial utility for each claimed invention to satisfy the utility requirement." MPEP 2107II(B)(1)(ii). Importantly, proof of efficacy in humans is **not** a requirement for credible therapeutic utility in humans. MPEP 2107VI.

Moreover, the Federal Circuit has reiterated that therapeutic utility sufficient under the patent laws is not to be confused with the requirements of the FDA with regard to safety and efficacy of drugs marketed in the United States. MPEP 2107.01 III.

> FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws. *Scott [v. Finney]*, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 [Fed. Cir. 1994)] Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating the incentive to pursue, through research and

> development, potential cures in many crucial areas such as the treatment of cancer.
>
> MPEP 2107.01 III (citing *In re Brana*, 51 F.3d 1560, 34 USPQ2d 1436 (Fed. Cir. 1995)).

The MPEP goes on to state that these general principles are equally applicable to the situations where an applicant has claimed a process for treating a human or animal disorder. *Id.*

Furthermore, the MPEP states:

> If reasonably correlated to the particular therapeutic or pharmacological utility, data generated using in vitro assays, or from testing in an animal model or a combination thereof ***almost invariably*** will be sufficient to establish therapeutic or pharmacologic utility for a compound, composition or process. A cursory review of cases involving therapeutic inventions where 35 U.S.C. 101 was the dispositive issue illustrates the fact that the Federal Courts are not particularly receptive to rejections under 35 U.S.C. 101 based on inoperability. Most striking is the fact that in those cases where an applicant supplied a reasonable evidentiary showing supporting an asserted therapeutic utility, almost uniformly the 35 U.S.C. 101-based rejection was reversed...Only in those cases where applicant was unable to come forward with ***any*** evidence to rebut a finding by the Office that the claimed invention was inoperative was a 35 U.S.C. 101 rejection affirmed by the court...MPEP 2107.03.

Importantly, evidence does not even have to be in the form of data from an art-recognized animal model for the particular disease or condition to which the asserted utility relates. Rather, data from any test that the applicant reasonably correlates to the asserted utility should be evaluated substantively. MPEP 2107.03 III. If one skilled in the art would accept the animal tests as being reasonably predictive of utility in humans, evidence from those tests should be considered sufficient to support the credibility of the asserted utility. MPEP 2107.07 III.

In the present case, Applicants previously have submitted data from the NOD mouse model showing that the presently claimed method resulted in (a) protection of pre-diabetic mice from becoming diabetic and (b) restoration of normoglycemia in all mice tested. (See Declaration of co-inventor Dr. Habib Zaghouani dated December 19, 2007, already of record). Not only is the NOD mouse model an art-recognized animal model for type 1 diabetes, but it has been characterized by Baxter and Duckworth (previously submitted) at page 452 as "the most

characterized and best-validated model of autoimmune diabetes; it is the **gold standard** for modeling aetiologic, immunological, pathological and genetic aspects of the disease" (emphasis added). Therefore, direct evidence of record supports a reasonable correlation between the evidence of utility of record and the disease to which the asserted utility relates (type 1 diabetes).

The Office Action states that "it appears that the only actual evidence of record supports the position that results of therapies in animal models cannot be used to predict the results of the same therapies in humans." OA at 6. Applicants respectfully disagree with this conclusion. First, as discussed in detail above, evidence of efficacy in humans is not required. Second, the utility requirement only requires a reasonable correlation between the activity in question and the asserted utility, not absolute certainty.

i. Couzin does not evidence failure of the NOD model to predict success in humans.

The Office Action points to a post-filing date lay paper by Couzin allegedly disclosing two failed attempts to treat human diabetes by administering insulin (oral and injected). As an initial matter, Applicants note that there is a significant difference between the apparent approach and compounds administered in studies discussed in that paper and the instant claims. Further, the Office Action characterizes Couzin as describing attempts to induce "tolerance to insulin." OA at 5. No indication is found in Couzin that the researchers were in fact attempting to induce "tolerance to insulin." Rather, the article suggests that insulin was selected because it was thought insulin could boost the number of T cells or that reducing glucose levels by supplying insulin lessened the stress on beta cells. Couzin at 1863, Col. 2. Therefore these alleged failed attempts to not appear to reflect failed attempts at inducing tolerance as characterized in the Office Action.

Notwithstanding the foregoing, the Office Action states that "both oral and injected forms of insulin could be used to induce tolerance in the NOD mouse", but subsequently failed in humans. OA at 5. Applicants note that at page 1863 Couzin indicates that earlier "rodent" and "preliminary human date" had suggested that it (injected insulin) could ***prevent*** diabetes. It is unclear from that passage whether the rodent model in question was the NOD model, whether tolerance to insulin was in fact induced and if so what the mechanism for oral and injected tolerance might have been. If additional evidence regarding the NOD model appears in Couzin, Applicants were unable to find it and respectfully request a citation to the appropriate page and

line. Moreover, even if the underlying studies discussed in Couzin were performed in NOD mice, that would appear to be even further evidence that the researches believed there was a "reasonable correlation" between the NOD model and type 1 diabetes in humans, even if oral or injected insulin treatment did not end up working in humans.

ii. Harrison touts the NOD mouse model's ability to predict success in humans.

The Office Action further quotes the following statement in Harrison's abstract as evidence that the NOD model is not predictive of therapy in humans: "This strategy [negative vaccination] is therapeutically effective in inbred rodent models but its translation in humans has failed to meet expectations." The Office Action, however, critically omits the sentence which immediately follows the quoted sentence that states: "This failure can be attributed to the use of suboptimal dosage regimens in end-stage disease, as well as other factors." Thus, Harrison's statement was not intended as an indictment of the NOD model itself or its predictive value in humans, but rather of the dosing and timing (end stage disease) of the studies being referred to. In fact, Harrison goes on to state that:

> ...the spontaneously diabetic non-obese diabetic mouse, which mimics human type 1 diabetes in many ways, ***has provided 'proof of concept' for negative vaccination***. Recent trials of a nasal insulin vaccine in humans at risk of type 1 diabetes ***provide evidence of tolerance induction as a basis for clinical efficacy***." (Harrison Abstract; emphasis added).

Harrison further states that:

> A prerequisite for development of a human therapeutic is demonstrable efficacy and safety in animal models. The NOD mouse has greatly contributed not only to our understanding of disease mechanisms but to the expectation that T1D is preventable. Autoimmune diabetes in the NOD mouse shares features with human T1D, including polygenic inheritance dominated by genes for antigen presenting molecules in the MHC, autoimmune response to (pro) insulin and GAD65, transfer of disease by bone marrow and a protracted pre-clinical phase. NOD mice respond to many immune and other interventions, but most of these prevent disease in only a proportion of mice, others only retard disease onset and some have no effect (and therefore are not reported). ***The NOD mouse has provided 'proof of concept' for islet-antigen-specific vaccination strategies, as a basis for human***

> ***trials to prevent T1D***. (Harrison, p.141., emphasis added; internal citations omitted)

Thus, according to the Harrison reference (relied on by the Office for the proposition that results in animal models can't be used to predict results in humans), results in the NOD model actually provide proof of concept that form the very basis for moving forward with human trials.

Lastly, Applicants expressly disagree with the Examiner's comments in the second paragraph at page 6 of the Office Action. In the Office Action dated August 24, 2007, the Office stated, in the context of a 35 U.S.C. 112, first paragraph utility rejection, that at the time prior to the instant application's filing date and based on the Legge 1998 publication "it is just as likely that the method of the instant claims would exacerbate disease as treat or prevent it." OA dated 08/24/2007, page 6. Applicants argued, in response to an obviousness rejection in that same Office Action, that this unpredictability was evidence of non-obviousness. (OA response dated 12/19/2007, page 12). The Office somehow attempts to interpret Applicant's argument as an agreement with the Office that the instant claims are not enabled by the instant specification. Applicants expressly traverse this conclusion as factually and legally misguided. The results of the presently claimed methods were unpredictable ***prior to*** Applicants' invention. However, Applicants enabled the present claims via filing of the instant patent application and priority documents thereto. These are two different inquiries altogether focusing on different timeframes and different legal frameworks.

Conclusion:

Applicants respectfully submit that they have more then met the burden of providing specific, substantial and credible utility for the presently claimed invention. The evidence of record (e.g. Harrison and Baxter) strongly supports the fact that the NOD mouse is the gold standard model for type 1 diabetes and is used as a basis for human clinical trials. Since a 35 U.S.C. 101 lack of utility rejection cannot be sustained, nor is a utility rejection under 35 U.S.C. 112 proper. Withdrawal if the instant 35 U.S.C. 112 utility rejection is therefore requested.

III. Rejection Under 35 U.S.C. 103.

Claims 1-5, 7, 13, 15-19 and 22-26 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over WO 98/30706 in view of Kaufman et al., J. Clin. Invest. Vol. 89 pp. 283-292 (1992) ("Kaufman"). Applicants respectfully traverse this rejection.

1. No *prima facie* case established.

To establish a *prima facie* case of obviousness under 35 U.S.C. § 103, the Office must first demonstrate that a prior art reference, or references when combined, teach or suggest all claim elements. See, *e.g., KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1740 (2007); *Pharmastem Therapeutics v. Viacell et al.*, 491 F.3d 1342, 1360 (Fed. Cir. 2007); *Abbott Laboratories v. Sandoz, Inc.*, 529 F.Supp. 2d 893 (N.D. Ill. 2007) and MPEP § 2143(A)(1). In addition to demonstrating that all elements were known in the prior art, the Office must also articulate a reason for combining the elements. See, *e.g., KSR* at 1741; *Omegaflex, Inc. v. Parker-Hannifin Corp.*, 243 Fed. Appx. 592, 595-596 (Fed. Cir. 2007) citing *KSR*. Further, the Supreme Court in *KSR* also stated that "a court ***must*** ask whether the improvement ***is more than*** the predictable use of prior art elements according to their established functions." *KSR* at 1740 (emphasis added). As such, in addition to showing that all elements of a claim were known in the prior art and that one of skill had a reason to combine them, the Office must also provide evidence that a reasonable expectation of success existed. MPEP 2143.02.

As will be discussed in detail below, Applicants respectfully submit that in the instant case, no articulation of the reasons why the claimed invention would have been obvious has been established with respect to the claims, no reasonable expectation of success existed at the time the instant application was filed, and each and every claim limitation is not disclosed in the prior art. As such, the asserted *prima facie* case of obviousness fails.

According to the office action, WO 98/30706 teaches the treatment of autoimmune disorders employing a humanized IgG2b chimeric protein wherein an autoantigen peptide is inserted into the D segment of a CDR3 loop. OA at 7. WO 98/30706 is silent as to GAD65, GAD1 and GAD2. Kaufman, on the other hand, is cited for its disclosure that GAD65 and GAD67 may be involved in IDDM via molecular mimicry with the coxsackievirus. Kaufman is silent as to GAD1 and GAD2.

A. No articulated reasoning supports the asserted obviousness rejection.

The Office Action indicates that one of ordinary skill in the art would have been motivated to select GAD65 as the autoantigen for use in the claimed invention given the teachings of Kaufman. As an initial matter, the claim amendments proposed herein define the protein fragment or peptide as "GAD2 represented by SEQ. ID NO 4." SEQ. ID NO 4 is not

specifically taught or disclosed in Kaufman or WO 98/30706, nor is there any articulated reasoning in the record that would have led one of ordinary skill in the art to modify WO 98/30706 so as to select SEQ ID NO 4 over any other peptide or fragment thereof derived from GAD65 or GAD67 or any other suspected diabetic autoantigen protein for that matter. Absent such articulated reasoning, no *prima facie* case of obviousness has been established.

The Office Action further states that the teachings of Kaufman "indicate that GAD65 was one of the few known IDDM autoantigens at the time of the invention." OA at 7. Applicant respectfully disagrees with this conclusion. Kaufman was published in 1992 whereas the instant application has an earliest claimed priority date of April 9, 2002. Therefore, Kaufman is not necessarily indicative of the state of known IDDM autoantigens nearly a decade after Kaufman's publication date. Applicants respectfully point out that many potential diabetes autoantigens were known or suspected as of the filing date of the instant invention including, without limitation, islet cell autoantigen 69, glutamic acid decarboxylase, islet tyrosine phosphatase ICA512/IA-2, heat shock protein 60, carboxypeptidase H, 38-kDa protein, peripherin, and gangliosides (*e.g.* GM 2-1 and GM3), *etc.* In addition to the proteins themselves, there are numerous possible peptides and fragments of the foregoing. Applicants submit that there is no articulated rationale in the record as to why a person of ordinary skill in the art would have, at the time the present invention was made, selected SEQ ID NO. 4 from among the numerous potential type 1 diabetes autoantigen peptides and protein fragments and combined it with the construct of WO 98/30706. As such, no *prima facie* case of obviousness exists.

The Office Action further states that "[r]egarding timing of administration of the Ig-fusion protein set forth in claims such as 3, 16, 17, etc., said timing would comprise only routine optimization which would fall well within the purview of one of skill in the art at the time of the invention." OA at 7. Applicants respectfully disagree with this statement as conclusory and unsupported by any evidence or rationale. According to the MPEP, the mere statement that the claimed invention is within the capabilities of one of ordinary skill in the art is not sufficient by itself to establish a *prima facie* case of obviousness without some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness. MPEP 2143.01IV. No such articulated reasoning with rational underpinning has been provided to support the Office's conclusion. As such, no *prima facie* case of obviousness has been established.

For at least the foregoing reasons, withdrawal of the instant rejection is therefore respectfully requested.

B. No reasonable expectation of success.

Even if one of ordinary skill in the art would have had some reason combine SEQ. ID NO. 4 with the construct of WO 98/30706 to prevent or delay the onset of type 1 diabetes, which is not admitted, such a person would not have had a reasonable expectation of success in preventing or delaying the onset of type 1 diabetes, particularly in a subject that had undergone insulin autoantibody seroconversion.

The Office Action relies on WO 98/30706 which discloses a fusion protein having the proteolipid protein (PLP) autoantigen inserted into the D segment of a CDR3 loop. PLP is an autoantigen associated with multiple sclerosis. Applicants respectfully submit that the multiple sclerosis test model used in WO 98/30706 (experimental allergic encephalomyelitis) is far different from the type 1 diabetes NOD mouse model used in examples within the instant application such that any success or failure shown in WO 98/30706 would not be at all predictive of success or failure of an Ig-GAD2 fusion protein in prevention or delay of type 1 diabetes as presently claimed.

Specifically, the relevant examples in WO 98/30706 (*e.g.* Examples I and XI) involve induction of an immune response with a known pathogenic peptide (PLP1) followed by treatment of the induced immune response with a slightly altered version of the very same peptide (PLP-LR) introduced in the form of a chimeric antibody immunomodulating agent. PLP-LR is an analog of PLP1 in which Trp144 and His147 are replaced with Leu and Arg, respectively. Therefore, in Examples I and XI of WO 98/30706, a disease state is induced with a known pathogenic peptide and then treated with a slightly altered non-pathogenic version of the very same peptide.

In stark contrast to those examples, the onset of type 1 diabetes in the NOD mouse model is a *spontaneous* event not triggered by administration of a known peptide antigen. Because no inducer peptide is known, it was completely unpredictable at the time the present invention was made which peptide antigen, if any, when incorporated into compositions disclosed in the instant application, would have any impact on type 1 diabetes, let alone delay or prevent that disease state. This is very different from the situation in WO 98/30706 in which the disease inducing peptide was known at the outset, and treatment was provided with a slight variation of the very

same inducer peptide. In view of these significant differences and the highly unpredictable area of art of the presently claimed invention, a person of ordinary skill in the art at the time the present invention was made would not have had a reasonable expectation of success in delaying or preventing type 1 diabetes according to the presently claimed methods. Applicants respectfully submit that the outcome of the presently claimed methods was highly unpredictable at the time the present invention was made.

Furthermore, one of ordinary skill in the art at the time the present invention was made would not have had a reasonable expectation that SEQ ID NO 4, selected from the numerous type 1 diabetes autoantigen peptides and protein fragments known or suspected at the time, would prevent or inhibit diabetes as presently claimed. There is no articulated rationale in the record for selection of any particular diabetogenic peptide or protein fragment, nor any indication why a person of ordinary skill in the art would have had a reasonable expectation of delaying or preventing type 1 diabetes in an IAA positive subject with any such peptide or protein fragment.

Finally, the Office Action states that "the ordinary skilled artisan would likely have developed a treatment employing a combination of unaltered and altered GAD peptides to avoid the problems that might have occurred due to the administration of individual peptides. Employing this strategy the ordinary skilled artisan would have had every expectation of success in developing an effective treatment." Firstly, as is discussed above, no such expectation would have existed given the fundamental differences between the experimental allergic encephalomyelitis and NOD models. Secondly, the presently claimed invention does not require use of a combination of unaltered and altered GAD peptides. Therefore, based on the Office's interpretation of WO 98/30706, that reference actually teaches away from the presently claimed invention.

For at least the foregoing reasons, a person of ordinary skill in the art would not have had a reasonable expectation of success of preventing or delaying the onset of diabetes according to the presently claimed methods. Withdrawal of the instant rejection is therefore respectfully requested.

C. Each and every claim limitation not disclosed in the prior art.

As amended herein, claim 1 and all claims depending there from specify that "the subject has undergone insulin autoantibody seroconversion prior to the administering step." This limitation is simply not disclosed in the prior art of record. Nor is SEQ ID NO. 4 disclosed in the

prior art of record. Because the prior art when combined does not teach each and every limitation of the instantly claimed invention, the asserted *prima facie* case of obviousness fails.

Conclusion:

Applicants respectfully submit that in the instant case, no articulated reason why the presently claimed invention would have been obvious has been established, no reasonable expectation of success existed at the time the instant application was filed, and each and every claim limitation is not disclosed in the prior art. As such, Applicants respectfully submit that the asserted *prima facie* case of obviousness fails.

IV. Obviousness Type Double Patenting Rejection.

Claims 1-5, 7, 13, 15-19 and 22-26 stand provisionally rejected under the judicially created doctrine of obviousness-type double patenting over claims 1-7 and 13-16 of U.S. Serial N0. 11/290,070 and claims 1-7 and 13-16 of U.S. 11/425,084. Applicants will address these provisional rejections upon resolution of the outstanding non-provisional rejections.

CONCLUSION

The application is believed to be in condition for allowance. Early and favorable considerations is respectfully requested. The Commissioner is hereby authorized to charge deposit account 02-1818 for any fees which are due and owing.

Respectfully submitted,

BELL, BOYD & LLOYD LLP

BY _____
David B. Fournier
Reg. No. 51,696
Customer No. 24573

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EXHIBIT K

ELSEVIER
DISEASE MODELS
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Autoimmune diseases and inflammation

Models of type 1 (autoimmune) diabetes

Alan G. Baxter*, Rowena C. Duckworth

Comparative Genomics Centre, James Cook University, Townsville QLD 4811, Australia

Type 1 (autoimmune) diabetes is caused by T-cell autoreactivity resulting in destruction of the insulin-producing pancreatic islet β cells. Its aetiology involves complex interactions between multiple genetic and environmental factors, its pathogenesis involves interactions between many leukocyte subsets and their associated cytokines and its complications affect nearly every organ in the body. Modelling the disease is, therefore, the subject of much effort and surprising ingenuity. In this article, we review established and emerging models of the disease and give an indication of their use for different types of study.

Section Editor:
Daniel Altmann – Human Disease Immunogenetics Group, Imperial College, London, UK

Autoimmune diabetes is now considered to be a prototypical example of a T-cell-mediated autoimmune disease. In recent years, there has been enormous progress in the analysis of the genetic and immunological mechanisms underlying this disease. In turn, this is leading to several new therapeutic strategies. These efforts have been helped considerably by the convergence of findings from clinical studies and rodent models, especially those based on the nonobese diabetic mouse strain. Here, Alan Baxter – an expert in clinical and experimental models of diabetes – reviews the currently available models.

Introduction

Diabetes associated with insulin deficiency varies widely in its clinical features. A small proportion of cases is characterised by abrupt onset at birth, or in the first few months of life, without good evidence of autoimmune responses. A larger group with atypical diabetes develops the disease later in life, often in middle age, and shows variable insulin dependence and many features of type 2 diabetes. This article is limited to the discussion of models of type 1 (autoimmune) diabetes, which is classically characterised by: (1) onset in childhood, puberty or early adulthood; (2) evidence of autoimmune activation (circulating autoantibodies, T cells, or both, with specificity for islets, β cells or their constituents); (3) severe insulin deficiency associated with low C peptide levels; and (4) if untreated, the biochemical sequelae associated with the resulting failure in glucose homeostasis (polyuria, polydipsia, weight loss, ketoacidosis, coma and death).

Type 1 diabetes

Autoimmune diabetes is a disease of complex aetiology, with large numbers of poorly characterised genetic and environmental risk factors and modifiers. The pathogenesis of the disease is almost certain to be autoimmune in nature because: (1) it is associated with autoimmune phenomena; (2) it showed evidence of immunological memory in diabetic patients that received human leukocyte antigen (HLA)-matched pancreas transplants [1]; and (3) diabetes was inadvertently adoptively transferred by transplantation of non-T-cell-depleted bone marrow [2]. Owing to the relatively inaccessible location of the pancreas and the presence of digestive enzymes in the exocrine tissue of the organ, only limited data are available from biopsies. Similarly, pathological surveys of pancreatic tissue in patients dying of type 1 diabetes are surprisingly scant. As a consequence, the understanding of the mechanisms of β cell destruction is heavily influenced by *in vivo* models of the disease.

*Corresponding author: (A.G. Baxter) alan.baxter@jcu.edu.au

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Table 1. Comparison summary table

	In vitro models	*In vivo* models	*In silico* models
Pros	Simple, inexpensive, scalable	Generally the best available	Flexible
Cons	Limited in application	Complex, difficult to control	Dependant on data training
Best use of model	Studies of pathogenesis	NOD mouse: studies of aetiology and pathogenesis (especially genetic and environmental interactions), development of prediction strategies and identification of putative therapeutic interventions. BB rat: physiological studies, testing therapeutic devices. Transgenic: studies of pathogenesis, identification of autoantigens	Prediction
How to get access to the models	Insulinoma available from Edward H. Leiter[a]; TCR transgenic mice available from Kathryn Haskins[b] and Pere Santamaria[c]	NOD mouse available from Jackson laboratory[d]; BB rat available from Taconic[e]; TCR transgenic mice available from Kathryn Haskins[b] and Pere Santamaria[c]	Peptide-binding model available from Vladimir Brusic[f]; generalized linear modelling tools available from SAS[g]
References	[3–8]	[9–23]	[24–27]

Abbreviations: BB, BioBreeding; NOD, nonobese diabetic; TCR, T-cell receptor.
[a] http://www.jax.org/staff/edward_leiter.html.
[b] http://www.uchsc.edu/immunol/faculty.html.
[c] http://www.med.ucalgary.ca/webs/irgcraininghome/.
[d] http://www.jax.org/.
[e] http://www.taconic.com/.
[f] http://idmc.i2r.a-star.edu.sg:8080/~vladimir/.
[g] http://www.sas.com/.

In vitro models

Although tissue and cell culture systems are unable to model the induction phases of the autoimmune response, they play a major role in the investigation of effector mechanisms of β cell destruction. Cultured insulinoma cells (e.g. NIT-1 [3]) and islets from mouse, rat or human [4] pancreata have been exposed to cytokines and immune effector molecules, such as Fas ligand and nitric oxide, to determine their susceptibility to killing or functional modification by these factors [5]. NIT-1 insulinoma cells can be obtained by contacting the Jackson laboratory (http://www.jax.org/) (Table 1).

An extension of these studies is the co-culture of islets, β cells or antigen-presenting cells pulsed with islet lysates, with various immune cell subsets (usually cloned T cells) from either mouse models or affected patients [6]. These approaches have been used to characterise the immune effector mechanisms of disease [7] and to identify putative autoantigens [8]. Currently, the most convenient source of clonotypic T cells for such studies is T cell receptor (TCR) transgenic mice.

In vivo models

NOD mice

The nonobese diabetic (NOD) mouse strain is the most characterised and best-validated model of autoimmune diabetes; it is the gold standard for modelling aetiological, immunological, pathological and genetic aspects of the disease. The most extensively used line is the NOD/Lt line, which is available from the Jackson laboratory. Both male and female mice of this strain spontaneously develop lymphocytic infiltrates into their pancreatic islets of Langerhans and, depending on housing conditions, up to 90% of female and 50% of male NOD mice succumb to diabetes caused by specific, T-cell-mediated, destruction of the insulin-producing β cells. As in the human disease, many genetic regions (currently more than two dozen) have been linked to diabetes, and environmental factors such as intercurrent infections seem to play a role in modulating tissue destruction, its biochemical sequelae, or both. Perhaps the most extraordinary aspect of the model is that there is some evidence that the same genetic risk factors that contribute to disease susceptibility in NOD mice also do so in humans. Several mouse diabetes loci have been mapped to locations syntenic to human diabetes susceptibility genes (*Idd1*, *Idd4*, *Idd5* and *Idd21*) and, in at least some cases, the homologous gene has been shown to be involved [*A* (GenBank accession numbers NM_010379 and X54425) and *Ctla4* (GenBank accession number NM_009843)] [9]. Indeed, NOD mice have an amino acid substitution at the same position in the gene homologous to the major histocompatibility complex (MHC) class II molecule DQβ, to which much of the HLA-linked disease susceptibility in humans has been attributed [10].

Some concerns have been raised about this model. Islet destruction occurs over a much briefer period in NOD mice than in humans; the infiltrates are much more pronounced in mice; the autoantibody specificities show some differences

[11]; the diabetes is at the severe end of the clinical spectrum; and, if the diabetes is not treated, death tends to occur from dehydration, rather than ketoacidosis. Recent scepticism over failed clinical trials of candidate therapies identified in the mice [12] is probably unwarranted, because, generally speaking, the trial protocols concerned did not compensate for limitations in clinical translation, such as dose escalation and narrow therapeutic windows, that were clearly identified by the preclinical studies.

Transgenic mouse models

One aspect of the complexity of the NOD mouse encompasses the processes required to generate a high frequency of autoreactive T cells bearing receptors with sufficient affinity to cause β cell destruction. A transgenic approach has been taken to eliminate this issue by inducing the transgenic expression of a TCR that is specific for native β cell autoantigens [receptor BDC2.5 – available from Diane Mathis' laboratory (www.hms.harvard.edu/dms/immunology/fac_mathis.html) [13]; receptor BDC6.9 – available from Kathryn Haskins' laboratory (http://www.uchsc.edu/immuno/faculty.html) [14]; receptor 4.1 [15] and receptor 8.3 – available from Pere Santamaria (http://www.med.ucalgary.ca/webs/irgtraining/home/) [15,16]], by inducing transgenic expression of a non-native antigen on the β cells, for which a high frequency of reacting T cells can be generated [e.g. the nucleoprotein of lymphocytic choriomeningitis virus – available from Matthias Von Herrath's laboratory (http://www.liai.org/) [17]], or by transgenic expression of both a TCR and a target antigen [e.g. the INS-HA TCR against influenza hemagglutinin under control of the rat insulin promoter – available from Harald von Boehmer (http://www.dana-farber.org/res/departments/cancerimmunology/) [18]]. The inherent simplicity of these models can offer distinct advantages over the NOD mouse in the study of accessory factors (such as molecular mimicry, effects of intercurrent infection, accessory molecules and cytokines) in the pathogenesis of disease. Because the TCRs involved are either CD4 or CD8 associated, it is possible to study the roles of these T cell subsets in isolation or in carefully titrated combinations.

In several other transgenic mouse models, insulin insufficiency is observed following transgenic expression (usually driven by the rat insulin promoter) of proteins in β cells in the absence of any other genetic or immunological manipulation. These particular models are probably artifactual because they do not show evidence of autoimmune causality.

Virally induced mouse models

Some viruses can induce insulin-dependent diabetes in wild-type (i.e. not genetically manipulated) mice. An example is the encephalomyelitis virus variant D, which causes islet destruction by tropism and direct cytotoxic effects. These models do not involve a major autoimmune component (reviewed in [19]).

Humanised mouse models

A concerted multi-investigator effort is underway to develop transgenic and knockout mouse models of the human immune system for the study of autoimmune diseases and transplantation issues. Various components of human responses are being transgenically introduced into mice, including HLA molecules (optimally in association with targeted deletion of native *H2* molecules), TCRs, CD4 and/or CD8, co-stimulator molecules (such as CD80, CD86) and complement inhibitors. Such systems can be used to study thymic selection of autoimmune T cells [20]. In some cases, the aim is to produce a human-like environment to foster the survival or development of human lymphoid cells following adoptive transfer of peripheral blood lymphocytes and/or bone marrow. In these cases, an immunodeficient mouse host (e.g. *scid* or $rag^{-/-}$) is used, often on a NOD background, because of a coincidental accumulation of several immune defects in this strain (for a review of these systems, see [21]).

BioBreeding rats

Like NOD mice, BioBreeding diabetes-prone (BBDP) rats spontaneously develop anti-islet autoantibodies and T-cell-dependent, ketosis-prone autoimmune diabetes. BBDP rats are available from Taconic (http://www.taconic.com/). A major advantage of BBDP rats over NOD mice is their larger size, which facilitates physiological experiments (e.g. draining of lymphatic ducts, intravital monitoring). Disadvantages include the relatively reduced number of immunological reagents (such as monoclonal antibodies and recombinant cytokines), the lack of genetically manipulated (gene-targeted mutant and transgenic) stocks and the presence of a severe T cell lymphopaenia. Comparison with the related, but non-lymphopaenic, BioBreeding diabetes-resistant strain revealed that the lymphopaenia in BBDP rats was largely attributable to a lack of regulatory T cells expressing the ART2 marker. Adoptive transfer of ART2$^+$ cells into BBDP rats prevents the onset of diabetes. An interesting quirk to this model is that diabetes can be induced in BBDR rats by several treatments, including infection with Kilham rat virus and immune activation with polyinosinic-polycytidylic acid [poly(I:C)] (see [22] and references therein).

Streptozotocin-induced diabetes

Streptozotocin is a broad-spectrum antibiotic with diabetogenic properties mediated by direct β cell cytotoxicity [available from Sigma-Aldrich (http://www.sigmaaldrich.com)]. A single dose of 200 mg kg^{-1} results in near-complete elimination of insulin production in a broad range of species, including mice. This model has no major autoimmune features, but is used widely to model the complications of disturbed glucose homeostasis associated with insulin insufficiency. Prior to the introduction of the NOD mouse and BioBreeding (BB) rat, a model of diabetes induced by repeated administration

of lower doses (30–40 mg kg^{-1}) was commonly used, in the belief that the associated islet infiltrates indicated an autoimmune origin for the ensuing β cell loss. Although this model has been largely discredited as a model of autoimmunity, it is still occasionally used in species for which other models are not available [23].

In silico models

Computer modelling of autoimmune processes, and indeed immune responses generally, is currently in its infancy [24]. Perhaps the most comprehensive model of the biochemical pathophysiological processes associated with type 1 diabetes published to date is the Archimedes diabetes model [25]. This model consists of a network of interrelated variables (e.g. plasma insulin level, glucose uptake by muscle and glucose production by liver) linked by differential equations describing the nature of the interactions between each variable. The variables used, and the existence of interactions between them, are selected by the investigators on the basis of current knowledge of biological systems. Output variables can be extremely complex, and include severity of symptoms and the presence of vascular complications. The model requires training on clinical data sets to derive each differential equation. It then iteratively generates a pool of virtual individuals with specific clinical and biochemical characteristics, from which subsets can be selected according to clinical trial criteria. The model provides good correlation with the results of data sets obtained from independent clinical studies. As currently configured, Archimedes does not mimic immune processes but, in principle, these too could be incorporated.

An alternative strategy is the application of a generalized linear model in which outcomes are defined and explanatory variables (risk factors or causes) that might or might not be relevant to the disease processes are nominated [26]. Training on extensive data sets is needed to generate the linear predictor, an equation that contains the explanatory variables, their weightings and arithmetic terms that combine them in additive or synergistic ways, and the transforming link function which maps the output of the linear predictor to an appropriate distribution. An important advantage of this model is that it is not necessary to determine which explanatory variables are important or to understand pathological processes to model them. A major disadvantage is the large size of the data sets required for training. Generalized linear modelling is now commonly used and implemented in many statistical software packages, including SAS (http://www.sas.com/).

Artificial neural networks have been used to model individual components of autoimmune processes. For example, Honeyman *et al.* [27] created a model to predict peptides that bind to the diabetes-associated MHC class II molecule HLA-DR4(*0401) to predict T-cell epitopes of the autoantigen tyrosine phosphatase IA-2 [available from Vladimir Brusic (http://sdmc.i2r.a-star.edu.sg:8080/~vladimir/)]. Predictions were validated by testing synthetic peptides for their binding to DR4 and their ability to stimulate T-cell proliferation in humans at risk for diabetes.

Conclusions

The best models of the complex aetiology of type 1 diabetes are, in order, the NOD mouse and the BB rat. These models are also useful for examining aspects of the pathogenesis of the disease, although many of the transgenic models also make important contributions. Diabetes induced by nonautoimmune processes (such as poisons or viral infection) is only really useful for modelling the complications of hypoglycaemia. Most *in vitro* and *in vivo* models address much more limited questions and are usually adapted to suit the exact requirements of the investigators. The major exceptions to this generalisation are the ongoing attempts to model whole individuals *in silico*, which to date have not successfully simulated immune processes.

The question of how well these models relate to human disease remains an open one. Generally, the study of type 1 diabetes in humans is complicated by the inaccessibility of the affected tissues and a poor association between pathological changes in peripheral blood and those occurring in the islets. Modelling the disease, therefore, has its greatest value in determining the avenues of prediction and intervention that are most worthy of attention in clinical studies.

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